EXPERIMENTAL CELL RESEARCH

Published under the auspices of

THE INTERNATIONAL SOCIETY FOR CELL BIOLOGY

EDITORS:

TORBJÖRN CASPERSSON · HONOR B. FELL · JOHN RUNNSTRÖM

FRANCIS O. SCHMITT · PAUL WEISS · RALPH W. G. WYCKOFF

VOLUME II · JUNE 1951 · NUMBER 2

ACADEMIC PRESS INC., PUBLISHERS, NEW YORK, N.Y.

EXPERIMENTAL CELL RESEARCH

EDITORS:

T. Caspersson, Department of Cell Research, Karolinska Inst., Stockholm 60, Sweden
Honor B. Fell, Strangeways Laboratory, Cambridge, England
J. Runnström, The Wenner-Gren Institute for Experimental Biology, Stockholm, Sweden
Francis O. Schmitt, Massachusetts Institute of Technology, Cambridge 39, Massachusetts
Paul Weiss, University of Chicago, Chicago 37, Illinois
Ralph W. G. Wyckoff, Laboratory of Physical Biology, National Institute of Health, Bethesda, Maryland.

EXECUTIVE COMMITTEE:

T. Caspersson, Honor B. Fell, J. Runnström and J. F. Danielli, King's College, London, W. C. 2, England.

ADVISORY BOARD:

H. Bauer (Wilhelmshafen), W. Bloom (Chicago), J. Brachet (Bruxelles), F. Buchthal (Copenhagen), A. Buzzati-Traverso (Verbania Pallanza), R. Chambers (New York), E. Chargaff (New York), J. F. Danielli (London), C. D. Darlington (London), J. N. Davidson (Glasgow), M. Demerec (Cold Spring Harbor, N. Y.), J. Engelbreth-Holm (Copenhagen), E. Fauré-Frémiet (Paris), A. Fischer (Copenhagen), A. Frey-Wyssling (Zürich), N. Fries (Uppsala), R. J. Gautheret (Paris), L. Geitler (Wien), G. Gomori (Chicago), A. Haddow (London), E. Hammarsten (Stockholm), B. Hanström (Lund), E. Newton Harvey (Princeton, N. J.), L. V. Heilbrunn (Philadelphia, Pa.), G. C. Heringa (Amsterdam), A. Hollaender (Oak Ridge, Tenn.), H. Holmgren (Stockholm), H. Holter (Copenhagen), J. Holtfreter (Rochester, N. Y.), P. Koller (London), F. Lehmann (Bern), P. L'Héritier (Paris), G. Levi (Torino), P. E. Lindahl (Uppsala), G. Lindeberg (Uppsala), C. C. Lindegren (Carbondale, Ill.), R. J. Ludford (London), S. E. Luria (Bloomington, Ind.), A. Lwoff (Paris), A. Mirsky (New York), G. Montalenti (Naples), H. Okkels (Copenhagen), O. Olivo (Bologna), A. W. Pollister (New York), J. Randall (London), W. J. Schmidt (Giessen), F. Schrader (New York), W. Seifriz (Philadelphia, Pa.), T. M. Sonneborn (Bloomington, Ind.), J. M. Sosa (Montevideo), J. Spek (Rostock), W. M. Stanley (Berkeley, Calif.), E. Suomalainen (Helsingfors), E. L. Tatum (Stanford University, Calif.), T. Teorell (Uppsala), A. Tyler (Pasadena, Calif.), W. Wilbrandt (Bern).

Subscriptions

EXPERIMENTAL CELL RESEARCH

will be published in four issues annually at $ 12.00 per volume. The price of one issue is $ 3.50. Please send your subscription orders to Academic Press Inc., 125 East 23 St., New York 10, N.Y.

A QUALITATIVE ANALYSIS OF THE AMINO ACIDS IN ISOLATED CHROMOSOMES

G. YASUZUMI and G. MIYAO

The Genetics Laboratory and the Anatomical Institute of Medical School, University of Osaka, Osaka, Japan

Received March 21, 1950

According to current views, the nuclei of the cells of animals and plants consist, apart from water and lipids, essentially of two substances, a basic protein (protamine or histone) and nucleic acid. These constituents are supposed to form a salt-like compound, known as nucleoprotein, which constitutes the so-called chromatin of the nucleus. Kirby (1) was not able to demonstrate basic amino acids in the salivary gland chromosomes by means of paper chromatography. The present authors succeeded, however, in demonstrating basic amino acids such as arginine and lysine in the chromatin threads isolated from carp erythrocyte nuclei by the same method (5).

Investigations on the amino acid component in chromatin threads isolated from human leucocyte nuclei and salivary gland chromosomes of *Drosophila melanogaster* will be presented below. The amino acids were estimated after hydrolysis of the material by means of paper chromatography. The results of this and of the preceding paper will allow a comparison between the amino acid composition of the chromosomes in man, carp and *Drosophila*.

EXPERIMENTS

Preparation of isolated salivary gland chromosomes of Drosophila melanogaster.—The salivary glands of *Drosophila melanogaster* (wild-type) were placed in a test tube containing 33 per cent liquor potassae for 10 minutes. The sample was centrifuged, the supernatant fluid decanted from the precipitate, and liquor potassae replaced by distilled water. The centrifuging was repeated in a cold room kept at about 1° C until the supernatant fluid became clear. The last precipitate was examined under a microscope, and was found to consist mainly of threads of various lengths which showed a positive reaction to Feulgen's staining. Thus, about 0.02 g of precipitate was obtained from 2 000 *Drosophila larvae*.

Preparation of isolated chromatin threads from human leucocytes.—Peripheral blood was obtained by venipuncture. The sample was immediately placed in a paraf-

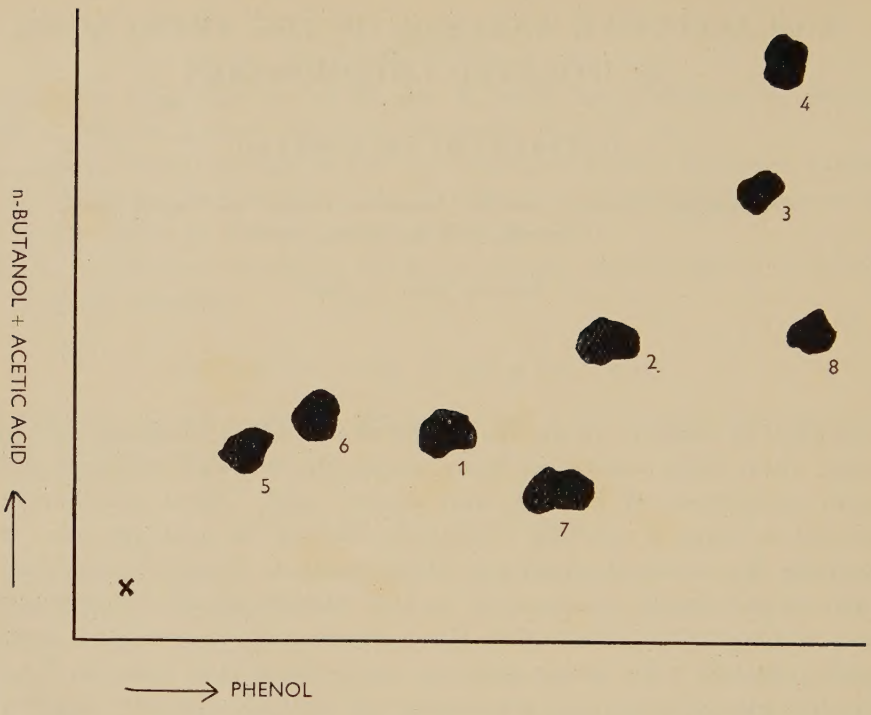


Fig. 1.

fin-coated test tube containing a small amount of powdered heparin. The tube was gently inverted to mix the blood with the anticoagulant. This blood sample was then put into a hematocrit tube with a capillary pipette, and centrifuged for a short time until the four major layers (plasma, thrombocytes, leucocytes and erythrocytes) were separated. The plasma layer and the thrombocyte layer were removed from the sample with a capillary pipette. The leucocyte layer was carefully put into 0.9 per cent salt solution. Then by means of Mirsky and Ris's technique (2) for isolating chromatin threads a sample of about 0.2 g per 100 ccm blood was obtained for examination. The isolated chromatin threads were examined by Yasuzumi and Mizuta with an electron microscope and an optic microscope. The procedure for the paper chromatogram was already described in a previous paper (5).

RESULTS

In the chromatin threads isolated from human leucocytes and salivary gland chromosomes of *Drosophila*, dissolved in distilled water and not hydrolyzed in NaOH or HCl, no trace of amino acid was found. Neither was tryptophane demonstrated in two samples hydrolyzed with NaOH.

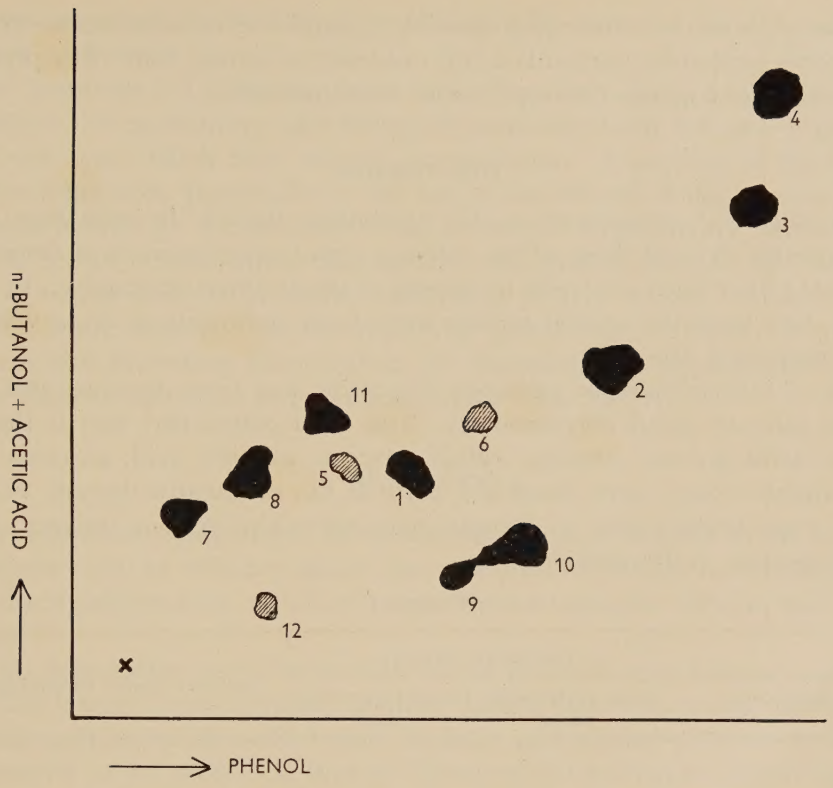


Fig. 2.

Fig. 1 is a tracing of a chromatogram for the demonstration of the various amino acids in the salivary gland chromosomes hydrolyzed with HCl.

By this method, the presence of the amino acids glycine, alanine, leucine, aspartic acid, glutamic acid, arginine and proline was demonstrated. Most of the spots showed a reaction to ninhydrin, and produced the typical purplish color, while one spot revealed the presence of proline by giving a yellowish color. The presence of arginine was confirmed beyond doubt, although its sensitivity to ninhydrin is commonly considered to be very low. This fact shows the existence of a large quantity of arginine in the salivary gland chromosomes.

Fig. 2 is a tracing of a chromatogram showing the amino acids which are contained in the chromatin threads of human leucocytes hydrolyzed with HCl. In this figure the presence of the amino acids glycine, alanine, valine, leucine, serine, threonine, aspartic acid, glutamic acid, lysine, arginine,

taurine (?) is shown. One spot, showing a purplish color after treatment with ninhydrin probably represents a still unidentified amino acid. The presence of lysine and arginine is very clearly demonstrated.

DISCUSSION

The chemical components of the chromatin threads isolated from carp erythrocytes (5) and those of the salivary gland chromosomes of *Drosophila virilis* (1) have been analyzed by means of paper chromatography. In table 1 the data from the quoted papers have been summarized along with the data presented above.

It is of interest that the presence of proline has been demonstrated only in the salivary gland chromosomes. The most noteworthy fact is that the amino acids glycine, alanine, valine, leucine, aspartic acid, glutamic acid and arginine have been identified both in the chromatin threads isolated from carp erythrocytes or human leucocytes and in the salivary gland chromosomes of *Drosophila*.

TABLE I

Amino acid or derivative	Chromatin threads		Salivary gland chromosomes	
	Carp erythrocyte nuclei	Human leucocyte nuclei		
Glycine	+	+	+	+
Alanine	+	+	+	+
Valine	+	+	+	+
Leucine	+	+	+	+
Phenylalanine	+	—	—	—
Serine	+	+	—	—
Threonine	+	+	—	—
Aspartic acid	+	+	+	+
Glutamic acid	+	+	+	+
Lysine	+	+	—	—
Arginine	+	+	+	—
Taurine (?)	+	+	—	—
Proline	—	—	+	+
Authors	Yasuzumi and Miyao			Kirby

While Kirby failed to demonstrate arginine in the salivary gland chromosomes, the present authors have ascertained that it is present in large quantities. Yasuzumi, Kita and Okazaki (4) demonstrated arginine in the euchromatin bands of the salivary gland chromosomes of *Drosophila virilis* with a

reagent prepared by dissolving p-dichloramido benzoic acid in normal sodium hydroxide.

The Stedmans (3) succeeded in isolating from fish sperm heads, not only a histone (or protamine) and desoxyribosenucleic acid, but also a protein of a new type which they named chromosomin. According to them this protein represents quantitatively the major constituent of the nucleus, and is characterized by its relatively large content of tryptophane. Mirsky and Ris (2) found three components, namely, desoxyribosenucleic acid, histone and a non-histone tryptophane-containing protein in the chromosomes isolated from nucleated erythrocytes and lymphocytes. However, neither this nor the preceding investigation (5) demonstrated tryptophane in the paper chromatogram of samples hydrolyzed with NaOH.

SUMMARY

The chemical components of chromatin threads isolated from human leucocytes and of salivary gland chromosomes of *Drosophila melanogaster* have been analyzed by means of paper chromatography with the following results.

1. No free amino acids have been found in these materials.
2. The amino acids glycine, alanine, valine, leucine, serine, threonine, aspartic acid, glutamic acid, lysine, arginine and taurine (?) have been demonstrated in the chromatin threads isolated from human leucocytes hydrolyzed in HCl.
3. In the salivary gland chromosomes of *Drosophila melanogaster* the amino acids glycine, alanine, valine, leucine, aspartic acid, glutamic acid, arginine and proline have been demonstrated in the hydrolyzate in HCl or NaOH.
4. The presence of tryptophane has not been ascertained.
5. Proline has been demonstrated only in the salivary gland chromosomes of *Drosophila melanogaster.*

This work was supported in part by a grant from the Science Research Found of the Ministry of Education.

REFERENCES

1. Kirby, B., *Proc. Natl. Acad. Sci.*, **34**, 561 (1949).
2. Mirsky, A. E., and Ris, H., *J. Gen. Physiol.*, **31**, 1, 7 (1947).
3. Stedman, E., and Stedman, E., *Nature*, **152**, 267 (1943).
4. Yasuzumi, G., Kita, T., and Okazaki, A., *Jap. of Genetics*, **23**, 59 (1948).
5. Yasuzumi, G., and Miyao, G., *Exp. Cell Res.*, **1**, 501 (1950).

L'ÉCHELLE DES INHIBITIONS FONCTIONNELLES DANS LA CELLULE VÉGÉTALE

P. GAVAUDAN et G. BREBION

Faculté des Sciences de Poitiers et Laboratoire Central des Services Chimiques de l'Etat, France

Received July 14, 1950

Les suspensions réversibles sous l'influence de diverses substances du déclenchement de la caryocinèse et des phénomènes mécaniques de son déroulement normal, des mouvements cytoplasmiques, de l'assimilation du gaz carbonique, de la synthèse de la chlorophylle sont des inhibitions fonctionnelles.

Le problème de la signification et du mécanisme de ces inhibitions est étroitement lié à celui de la narcose, ce terme étant pris dans le sens particulier de l'inhibition qui affecte plus spécialement les cellules nerveuses.

C'est l'action élective exercée par les substances du type de la colchicine qui a conduit à envisager dans son ensemble le problème des inhibitions fonctionnelles dans la cellule végétale. En effet, les recherches de l'un de nous en collaboration avec Dode et Poussel (5) ont montré l'existence d'un certain parallèlisme entre le déclenchement des troubles de la caryocinèse et les phénomènes narcotiques proprement dits provoqués par des substances du type des hydrocarbures cycliques: l'acénaphtène, par exemple. Gavaudan (4) provoque la narcose des batraciens et l'inhibition des mouvements de l'intestin isolé de lapin. De plus, les conditions physico-chimiques dans lesquelles les narcotiques indifférents provoquent soit la narcose nerveuse, soit la perturbation de la caryocinèse sont très semblables. On a constaté tout d'abord qu'en première approximation ces substances provoquaient ces inhibitions de types si différents pour des valeurs voisines de leur activité thermodynamique (I). Levan et Östergren (6) indépendamment de Gavaudan, Dode et Poussel (5) ont également fait remarquer que l'on pouvait considérer les perturbations de la caryocinèse comme une sorte de narcose puisque tous ces phénomènes suivaient la règle lipoïdique d'Overton-Meyer.

Gavaudan, Dode et Poussel (5) ont essayé de généraliser cette théorie narcotique de la perturbation de la caryocinèse en considérant ce phénomène et d'autres inhibitions fonctionnelles produites par les narcotiques dans la cellule végétale comme autant d'expressions diverses de la narcose.

Ils ont initialement admis le point de vue de Claude Bernard qui, le premier, avait tenté de relier entre eux les effets multiples produits par les narcotiques: inhibition de la fonction chlorophyllienne, suspension de la germination des graines, paralysie nerveuse.

Nous avons donc étudié systématiquement l'action des substances telles que la colchicine, l'acénaphtène et les autres carbures ou dérivés sur la photosynthèse et la synthèse de la chlorophylle dans le but de vérifier la généralité du parallèlisme entre le déclenchement des perturbations de la caryocinèse et les diverses inhibitions fonctionnelles.

Dans une première série d'expériences, nous avons observé que le benzène et les composés analogues comme le chlorobenzène, le nitrobenzène, le benzoate d'éthyle inhibaient la photosynthèse des feuilles de *Triticum Vulgare* à des valeurs d'activité thermodynamique de l'ordre de grandeur de celles qui produisent les anomalies de la caryocinèse.

On observait avec certaines substances une concordance nette entre les deux types d'inhibition. Toutefois, cette concordance ne s'étendait pas à l'acénaphtène et à la colchicine qui se montraient sans action sur la photosynthèse.

Brebion (2) a souligné un peu plus tard le parallèlisme quantitatif de l'action des narcotiques indifférents; non seulement avec des substances apolaires comme le benzène, le chloroforme, mais aussi avec des substances polaires comme les alcools.

ACTIVITÉS THERMODYNAMIQUES

A. *Inhibitions par le benzène*

Photosynthèse	*Scenedesmus*	0,40
	Elodea	0,33 à 0,50
	Triticum	0,60
	Triticum en phase gazeuse	0,33
Caryocinèse	*Triticum*	0,16 à 0,33
Système nerveux du tétard	Grenouille	0,16

B. *Inhibitions par l'alcool methylique*

Photosynthèse	*Elodea*	0,041
	Scenedesmus	0,041
Caryocinèse	*Triticum*	0,040
Mouvements cytoplasmiques de cellules végétales		0,041
Système nerveux du tétard		0,015

Dans des types cellulaires aussi différents que l'*Elodea* et le *Scenedesmus* la photosynthèse est inhibée à peu près dans les mêmes conditions par les narcotiques indifférents. On relève cependant la particularité suivante pour *Scenedesmus* et les alcools de la série homologue: alors que l'activité thermodynamique inhibant la photosynthèse chez l'*Elodea* monte de quelques centièmes à quelques dixièmes en passant du terme en C_1 au terme en C_8, elle demeure de l'ordre des centièmes dans le cas du *Scenedesmus*. Cette différence de sensibilité reliée au problème général de la montée de l'activité dans les séries homologues est cependant relativement peu importante et est étudiée ailleurs.

Le parallèlisme entre les diverses inhibitions fonctionnelles se montrait donc assez satisfaisant mais le cas de l'acénaphtène et de la colchicine montrait qu'il n'était pas possible de considérer la narcose sous un aspect unitaire. La fonction chlorophyllienne se montrait plus résistante à l'action de certains agents que ne l'étaient les phénomènes mécaniques de la caryocinèse par exemple.

On aurait pu, à la rigueur, supposer une mauvaise pénétration de la colchicine dans l'*Elodea* mais ceci n'était guère soutenable puisque les expériences de Dangeard, Biraben et Doutre (3) avaient montré que cette substance y active les mouvements cytoplasmiques. De nouvelles recherches et une interprétation plus large des résultats devenaient nécessaires.

Nous avons fait porter nos nouveaux essais sur les feuilles de *Triticum Vulgare* de façon à travailler sur le matériel déjà utilisé pour déterminer les seuils d'action perturbatrice de la caryocinèse.

Nos mesures de photosynthèse et celles de Brebion avaient été toutes effectuées au moyen d'un appareillage de Warburg spécialement équipé pour ces déterminations et qui a fait l'objet d'une description. Nous avons modifié cette méthode pour l'appliquer à la mesure de la photosynthèse des végétaux aériens afin de travailler dans des conditions plus normales avec un matériel tel que les feuilles de *Triticum*; cette méthode a été aussi décrite; Wassink (10) a utilisé également la méthode de Warburg pour l'étude de la photosynthèse des plantes supérieures mais en milieu liquide. Nous avons utilisé des fragments de feuilles de blé préalablement imprégnées sous vide par des solutions de colchicine ou d'acénaphtène. Dans ces conditions assurant une meilleure pénétration, la colchicine et l'acénaphtène ne se sont pas montrés inactifs ou à peu près comme dans nos premiers essais.

De plus, nous avons étudié parallèlement sur *Triticum vulgare* l'inhibition de la synthèse de la chlorophylle. On sait depuis les expériences anciennes

de Teodoresco et Coupin que les anesthésiques inhibent la formation de la chlorophylle chez les plantules étiolées reportées à la lumière.

Nous avons utilisé des plantules de blé germé à l'obscurité à une température constante de 20° et par conséquent étiolées. Les expériences ont été réalisées de deux façons:

1) Certaines expériences ont été faites sur plantules entières étiolées en introduisant dans les récipients de culture le narcotique volatil en solution huileuse. La solution huileuse (huile de ricin) était contenue dans un godet fixé à la base du récipient de culture. On réalisait de cette façon une atmosphère contenant le narcotique volatil à une activité thermodynamique connue grâce à la courbe de la tension de vapeur en fonction de la composition du mélange.

2) Certaines expériences ont été faites sur des feuilles isolées de blé contenues dans des tubes clos. La base des feuilles était immergée dans le liquide essayé, solution de colchicine, d'acénaphtène, etc. . . .

Nous avons donc imprégné les fragments de feuilles ou les feuilles sous vide en les immergeant dans les solutions de colchicine ou d'acénaphtène comme dans les expériences sur l'inhibition de l'assimilation chlorophyllienne. Nous avons ainsi observé une certaine inhibition du verdissement par la colchicine et l'acénaphtène.

Notons que dans ces expériences, le temps d'étiolement joue un grand rôle et que, dans la plupart des cas, nos expériences ont été effectuées après des temps de germination de 12 à 16 jours à l'obscurité. Avec les substances peu actives comme la colchicine et l'acénaphtène, l'inhibition est d'autant plus manifeste que le temps passé à l'obscurité est plus considérable.

Nous avons comparé le pouvoir inhibiteur qu'exercent la colchicine, l'acénaphtène et le benzène sur les fonctions suivantes de la cellule végétale (blé): croissance, déclenchement de la caryocinèse et germination, mécanisme de la caryocinèse, synthèse de la chlorophylle, assimilation du gaz carbonique.

Nous distinguons ici l'action inhibitrice sur le déclenchement des cinèses de l'action exercée sur les processus mécaniques de la caryocinèse parfois nommée action stathmocinétisante, mitoclasique, etc. . . . Certains auteurs (1) nomment la première action: inhibition préprophasique, nous la nommerons action mitostatique. Il ne s'agit pas là, comme le pensent certains auteurs, d'un phénomène nouveau. Ce phénomène est étroitement lié à l'inhibition de la germination observée dans la classique expérience de Claude Bernard avec les graines de cresson alénois et l'éponge imbibée d'éther. Dès 1925, P. A. Dustin observait les effets mitostatiques de la trypa-

flavine et à la même époque Politzer (8) notait que le rouge neutre pouvait empêcher l'entrée en prophase dans les cellules de la cornée des larves de *Salamandra maculosa*. Politzer montra également en 1931 que les narcotiques empêchaient l'entrée en division des cellules. Ce phénomène a toutefois été antérieurement négligé par Gavaudan dans les conditions d'une étude entièrement vouée à la description des effets exercés sur le mécanisme de la caryocinèse. Ces derniers effets seront désignés sous le terme de cinétoclasiques.

	Colchicine Concentration	Acénaphtène Activités	Benzène Activités
Croissance de la plantule de *Triticum*	1/20.000 nette inhibition 1/1000 très forte	saturation inhibition plus faible que la colchicine à 0,001	0,10—0,15 inhibition totale
Action mitostatique	faible à 1/1000	plus forte que la colchicine	totale à 0,10
Action cinétoclasique	1/20.000 très forte	0,20 très forte	0,05
Synthèse de la chlorophylle	1/1000 } Effets incomplets 1/4000 }	solution saturée inhibition faible	0,20 inhibition totale
Assimilation chlorophyllienne	1/1000 50 % inhibition 4/1000 complète	solution saturée 60 % inhibition	0,30 inhibition totale

Nous avons observé qu'il existe un décalage entre l'inhibition du déclenchement de la caryocinèse (action mitostatique), l'action cinétoclasique, les inhibitions de la synthèse de la chlorophylle et de l'assimilation chlorophyllienne par la colchicine et l'acénaphtène.

Le benzène agit d'une façon beaucoup plus homogène sur les différents phénomènes et dans une marge plus étroite d'activité thermodynamique, c'est-à-dire de 0,05 à 0,30. La colchicine inhibe encore la caryocinèse à 1/20,000 alors qu'à 1/1000 elle n'inhibe la photosynthèse qu'incomplètement. Il faut remarquer que la colchicine et l'acénaphtène avec lesquels s'observe le décalage maximum de sensibilité des diverses fonctions sont des toxiques de type différent d'après la classification thermodynamique (5): la colchicine étant du type chimique et l'acénaphtène du type physique. Ceci montre bien que dans les deux cas un certain degré de lésion du cytoplasme ou de certains récepteurs intracellulaires n'est pas atteint. Dans une cellule soumise à l'action de la colchicine, il n'existe pas un état généralisé de narcose du cytoplasme et de toutes ses fonctions. Dans la cellule colchicinée, on

sait que la synthèse des acides nucléiques se poursuit longtemps puisque de 16 dans l'*Allium cepa* le nombre de chromosomes peut être porté à plus de 1000 à la suite de 6 cycles de multiplication chromosomique non suivie de division cellulaire. La synthèse des gènes n'est pas non plus affectée puisque les particularités héréditaires sont transmises normalement dans les mutants polyploïdes obtenus avec cette substance. La synthèse de la chlorophylle est également résistante à l'action de la colchicine. Toutes ces synthèses dépendent donc de processus qui ne sont pas altérables exactement dans les mêmes conditions que d'autres processus tels que les remaniements protéiques qui conduisent à la formation des longues chaînes moléculaires que l'on suppose exister dans le fuseau de caryocinèse.

Les diverses fonctions ne sont pas également sensibles à l'action des divers agents inhibiteurs.

L'expérience grossière nous montre d'ailleurs que certaines substances comme l'acénaphtène ne peuvent provoquer la mort de la cellule même à saturation, contrairement au benzène qui se montre capable de tuer après une action des vapeurs à une activité de quelques dizièmes.

Si l'hypothèse du parallèlisme entre la narcose nerveuse et la production des phénomènes cinétoclasiques demeure exacte dans une certaine mesure, il n'est pas possible toutefois de donner au terme de narcose une signification univoque comme pensait pouvoir le faire Claude Bernard. Les électrophysiologistes ont récemment montré que la narcose nerveuse n'est pas un phénomène simple. Il n'est donc pas possible de prendre la narcose nerveuse comme système de référence.

Le fonctionnement du nerf est accompagné de manifestations électriques importantes qui permettent d'établir certaines caractéristiques de la cellule nerveuse. On distingue l'amortissement, c'est-à-dire l'aptitude à la réponse propagée (facteur de freinage), la hauteur du « spike » (c'est-à-dire l'importance de la réponse électrique), le potentiel de membrane.

Les électrophysiologistes Monnier (7), Posternak, Mangold et Laget (9) ont montré que les alcools de la série homologue pouvaient agir d'une façon qualitativement différente pour modifier ces caractéristiques. On observe des variations opposées de l'amplitude du spike et de l'amortissement. Il devient évident que ce que nous nommons globalement narcose et excitation par deux termes distincts représente la résultante de la combinaison de facteurs différents et opposés intervenant dans la même cellule.

Nous avons affaire à des collections de phénomènes élémentaires. L'organisation de ces collections de phénomènes donne des résultantes que nous nommons narcose ou excitation. Les narcotiques permettent de désynchroni-

ser ou de dissocier les fonctions élémentaires. L'unité d'un processus narcotique omnivalent dans toutes les cellules devient très douteuse.

Ceci nous explique pourquoi des substances comme l'acénaphtène ou la colchicine affectent beaucoup moins les processus de photosynthèse, de synthèse de la chlorophylle ou des acides nucléiques que ne le font des substances comme le benzène, tout en inhibant fortement le mécanisme de la caryocinèse.

Ceci est a rapprocher aussi des anciennes expériences de Medes et Mc Clendon dans lesquelles ces auteurs ont observé une augmentation de la respiration et des mouvements cytoplasmiques pour des concentrations narcotiques diminuant la photosynthèse. Soulignons encore que, d'après les expériences de Dangeard et ses collaborateurs (3) sur l'*Eloda*, la colchicine à 1/1000 accélère les mouvements cytoplasmiques alors qu'elle a plutôt tendance à diminuer la photosynthèse dans nos expériences sur le même matériel.

RÉSUMÉ

L'analyse comparative des inhibitions fonctionnelles par les narcotiques et les substances cinétoclasiques montre:

1° que ces inhibitions peuvent se produire par divers mécanismes.

2° que ces inhibitions peuvent être relativement indépendantes les unes des autres.

3° que les narcotiques indifférents; exemples: benzène, chloroforme, acénaphtène, agissent dans une marge d'activité étroite pour diverses fonctions; ceci souligne le rôle important joué par des structures communes, probablement du type des cénapses lipoprotéiques.

La comparaison des inhibitions fonctionnelles par les narcotiques (inhibition de la caryocinèse, de la photosynthèse, la narcose nerveuse, etc. . . .) indique une certaine indépendance de ces phénomènes en soulignant toutefois l'importance probable de l'altération de substances lipo-protéiques.

LITTÉRATURE CITÉE

1. Amato, F., *Caryologia*, **1**, 109 (1949).
2. Brebion, G., *Gallica Biol. Acta*, **1**, fasc. I, 24 (1948).
3. Dangeard, P., Biraben, J. et Doutre, L., *Compt. Rend. Soc. Biol.*, **141**, 1055 (1947).
4. Gavaudan, P., thèse Doctorat Etat, *Pharmacie*, Paris, 1945.
5. Gavaudan, P., Dode, M., et Poussel, H., *Mém. Ind. Chim. Etat*, **32**, 388 (1946).
6. Levan, A., et Östergren, G., *Hereditas*, **29**, 381 (1943).
7. Monnier, M., Colloque International sur la narcose, Paris, 1950.
8. Politzer, M., Pathologie der Mitose, Berlin, 1934.
9. Posternak, T., Mangold, E., et Laget, P., Colloque International sur la narcose, Paris, 1950.
10. Wassink, E. C., *Enzymologia*, **1**, 12 (1946).

SPECIFIC INHIBITION BY MESO-INOSITOL OF THE COLCHICINE EFFECT ON RAT FIBROBLASTS[1]

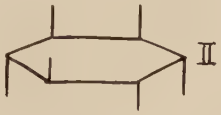
MARGARET R. MURRAY, HELENA H. de LAM and ERWIN CHARGAFF

Departments of Surgery and of Biochemistry, Columbia University, New York

Received July 26, 1950

INTRODUCTION

In a previous study in which one of these writers took part (3) it was shown that *meso*-inositol (I) exerted a remarkable cytological effect in the root-tip cells of *Allium cepa:* it blocked, under proper conditions, the arrest of nuclear division in the metaphase, produced by mitotic poisons such as colchicine and γ-hexachlorocyclohexane (Gammexane).[2] Another inositol isomer, namely *d*-inositol (II), and also *D*-sorbitol were ineffective.



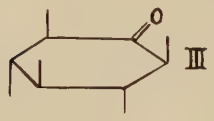
These findings were of interest, since they appeared to offer an insight into the wide-spread and hitherto largely unexplainable biological significance of *meso*-inositol. As one of the possible explanations of the activity of this inositol isomer the hypothesis was discussed that *meso*-inositol, or more probably a derivative of it, was in some way connected with the formation of the spindle.

It seemed desirable to extend these observations to animal cells in an experimental arrangement suited to a quantitative and statistical treatment of the results and to the investigation of a larger number of control substances. In the present study colchicine was the mitotic poison employed. In addition to several sugars and related substances the following keto derivatives of

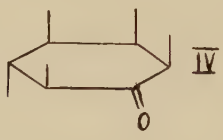
[1] This work was supported by grants from the American Cancer Society and the Damon Runyon Fund.

[2] It should, however, be noted that other studies, carried out in a merely qualitative fashion, claim to have been unable to confirm the effectiveness of *meso*-inositol (2, 4).

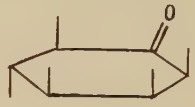
the inositol group were also tested: *meso*-inososе (III) and *d, l-epi*-inososе (IV and V).



III



IV



V

The tissue culture method seemed to offer the best opportunity for direct application of the chemical agents and for continuous observation of whole (unsectioned) cells. Of the various tissues of the rat which have been used as standard culture material in this laboratory, subcutaneous areolar tissue from adults seemed most advantageous for these purposes, since it can afford a source of fibroblasts in which the division rate is relatively low and steady, not varying greatly over a selected 24 hour period. It is also possible to obtain, from subcutaneous areolar tissue, cultures that are relatively pure genetically as regards tissue type. It does not seem possible, however, to insure physiological uniformity among different series of cultures derived from different donors; in media of the same composition by formula (though not necessarily from the same batch), tissue from one animal may show a growth rate more than twice that from another (see Graph II). Because of the prohibitive amount of time, labor and material that would be required to derive from a single strain the large number of cultures used in these experiments, it was necessary to use strains from several different donors. However, for a single experiment only one strain was used, and within each experiment variations among individual cultures were controlled by an arrangement described later. When different experiments were combined in one curve or table (as in Graph I, Tables XI and XII) it was first ascertained that they had been performed on strains with approximately equal growth rates. For a general discussion of the statistical treatment of mitosis the reader is referred to Hoffman (6), and to Love (7) for an application of the tissue culture method to the quantitative study of mitosis.

MATERIAL AND METHODS

Fibroblast cultures derived from the subcutaneous areolar tissue of adult rats were carried for 15 days or longer in roller tubes, with 1 passage, and were then transferred to Maximow slides which were thought best adapted to slow-growing adult tissues. Here, when they were about to reach their combined maximum area and growth rate—usually after 5 days—they were exposed to the experimental agents introduced

in the feeding solution. The regular feeding solution was composed of equal parts of human placental serum, beef serum ultra-filtrate (supplied by Microbiological Associates, Coral Gables, Florida) and 50 per cent chick embryo extract. This was used in the first feeding of the slide-cultures after explantation. At the time of an experiment two more parts were added, thereby producing a mixture of five equal parts in which an experimental agent represented 1 part in 5, or 20 per cent. Where two agents were used (for example colchicine and meso-inositol), each represented 20 per cent, making a total of 40 per cent. When only one compound was used, the second was replaced with a buffered physiological saline solution (13), Simms' X–7. In saline controls, both experimental agents were replaced with saline, which thus amounted to 40 per cent of the total feeding solution. The experimental formula expressed in per cent was, therefore, as follows:

Experimental Feeding Solution

Placental serum	20 per cent
Serum ultrafiltrate	20 » »
50 per cent embryonic extract	20 » »
Experimental agent 1 (or saline)	20 » »
Experimental agent 2 (or saline)	20 » »
	100 per cent

Each experimental agent therefore, after being introduced into the medium, was actually applied to the cultures at $\frac{1}{5}$ the concentration listed in Graph II.

Tube and slide cultures were incubated at 37° C. The pH of the experimental feeding solutions as indicated by phenol red (present in the medium as 1 cc. sat. aq. sol. per liter), was not affected by the experimental agents in the concentrations used.

Solutions of the experimental agents were sterilized by filtration through a Corning sintered glass filter, with the exception of the inositols, which were autoclaved for 15 minutes. In order to minimize any disturbance of the osmotic equilibrium by the experimental agents, they were made up in aqueous stock solutions at 100 times the molar concentration in which they were to be used, and these stock solutions were then diluted to the proper concentration by the addition of 99 parts of physiological saline.

Since it was found in preliminary experiments that the maximum colchicine effect was exerted on this tissue within 20 hours after the introduction of the agent, the cultures were habitually fixed at this time in Helly's formalin-mercuric-chloride-bichromate mixture and stained with Harris' haematoxylin. Counts were then made (using a Howard ocular grating) of the total number of cells and the number of cells in mitosis, in 4 low-power fields in the outgrowth from each culture. These fields were determined by north, south, east and west moves of the mechanical stage to the periphery of the outgrowth, using the explant as center. The mitotic index was computed by dividing the number of cells in division by the total number of cells.

Since it was impractical to obtain sufficiently large numbers of cultures for these experiments from old and therefore very uniform strains, the following method of handling was adopted: Roller tube cultures were allowed to grow until each one covered a rather large area. When they were to be transferred to slides in preparation

for an experiment, each culture was divided into 4 approximately equal parts. Since each experiment usually involved 4 groups of slides to which different agents were applied, one fourth of each roller tube culture was placed in each of the experimental groupings. Thus, though there might be considerable variation in mitotic rate between different cultures in a single group, the totals for the members of each group were comparable.

EXPERIMENTS

Effective Concentrations. In preliminary experiments the effects of colchicine in 10^{-4}, 10^{-5}, $10^{-6} \times 5$, $10^{-6} \times 2$ and 10^{-6} molar solutions, introduced in the proportion of 20 per cent of the feeding mixture, were tested on this material. Colchicine 10^{-6} produced little if any mitotic arrest and colchicine 10^{-4} appeared to be generally toxic. All the other concentrations showed in different degrees the specific colchicine effect, that is, a marked blocking of mitosis in metaphase. The action of colchicine $10^{-6} \times 2$ is little different quantitatively from that of $10^{-6} \times 5$. Since colchicine 10^{-5} produced the most marked mitotic arrest without general toxicity, it was decided to adopt this as the standard colchicine concentration. (See Tables I–IV.)

In their work on the onion root-tip, Chargaff, Stewart and Magasanik (3) found 10^{-3} molar to be the most effective concentration of *meso*-inositol relative to colchicine 10^{-5}. In rat fibroblasts the present writers have arrived at the same conclusion. *Meso*-inositol alone in concentrations of 10^{-2} or 10^{-3} does not produce any striking effects on the cells exposed to it, though they appear slightly healthier, stiffer and more refractive than the controls. *Meso*-inositol 10^{-3} is as effective as 10^{-2} in blocking the colchicine action. (Tables I–IV.)

Period of Exposure. Assuming that these compounds actually enter the cell, and exert their effects from within, it was thought that if the administration of *meso*-inositol preceded the exposure to colchicine a more marked blocking action might be produced. Consequently, *meso*-inositol was applied to the cultures 24 hours, 5 days and 13 days, respectively, before the application of colchicine (See Tables II, III, IV). A period of pre-treatment does not increase, but decreases the effects of *meso*-inositol, as compared with the simultaneous application of the two compounds. When *meso*-inositol has been applied as long as 13 days to the cultures, the mitotic index may be as much as twice that of the saline controls. The cells in a medium containing *meso*-inositol alone in these concentrations appear rather rounded and viscous; with cells in this state the process of mitosis may take a longer time than in the controls. Since the area of outgrowth in *meso*-inositol is not substantially greater than in the controls, it is probable that *meso*-inositol by itself does not actually increase the growth rate or reduce the interkinetic period.

Though *meso*-inositol in its most favorable concentration does not wholly block the action of colchicine on rat fibroblasts, it invariably reduces the mitotic index substantially. The figure for colchicine + *meso*-inositol ranges from one-fourth to one-half that of colchicine alone (see Graphs and Tables).

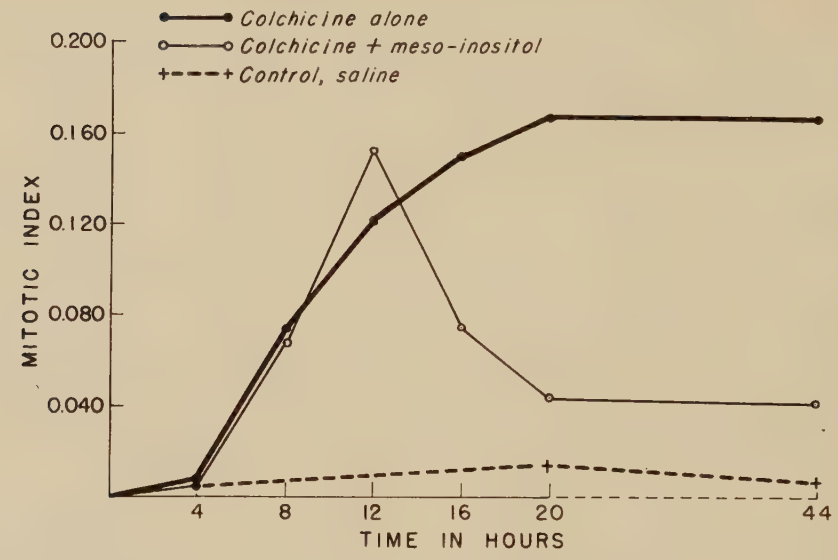
Action of Related Compounds. To test the specificity of the inhibition by *meso*-inositol of the colchicine effect, the following compounds were employed in a series of comparable experiments; *d*-inositol (differing from *meso*-inositol only in the sterical position of an OH group), *D*-sorbitol, ribose, sucrose, glucose, *meso*-inosose and *epi*-inosose. None of these has any special effect on cultured cells when used alone, nor does any of them, used together with colchicine, produce any blocking of the colchicine effect on mitosis. (See Graph II, Tables V–X.)

Time Sequence of Action. Within 20 hours after the simultaneous application of colchicine 10^{-5} and *meso*-inositol 10^{-3} the effects of both compounds are complete. There seem to be no changes during the next 24 hours, the longest time such an experiment has been run. It therefore seemed desirable to determine the relative time sequence in which these effects were exerted, in the interval between the exposure of the cells to the experimental agents and the time of maximum effect.

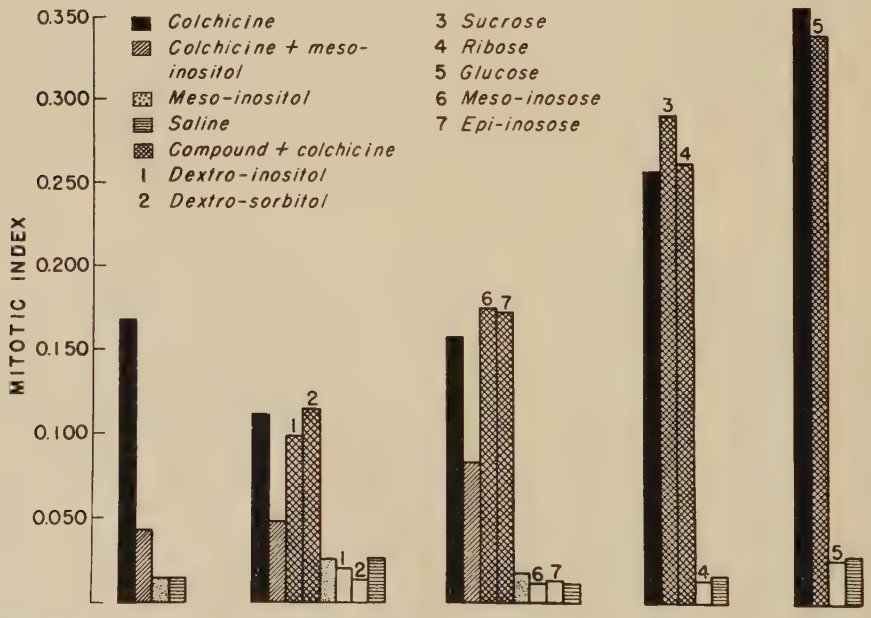
Consequently, a series of cultures was separated into 2 groups to which colchicine alone and colchicine + *meso*-inositol were applied, respectively. The whole series was then divided on a vertical scale into 4 parts, which were fixed after 4, 8, 12, and 16 hours, respectively. (See Tables XI and XII, and Graph I.) By this approach it was shown that *meso*-inositol has no effect in blocking the action of colchicine during the first 12 hours, but at that point it begins to reverse the colchicine action, producing a rapidly descending curve between 12 and 16 hours. At 20 hours (the standard fixation time) the descending curve reaches its lowest point. Thus it would appear that after a temporary metaphase arrest, the presence of *meso*-inositol must allow from one-half to three-quarters of the mitoses to proceed to completion.

RESULTS — TABLES, GRAPHS

The principal results obtained in the course of this study are assembled in Tables I–XII and in Graphs I and II. As has been explained above, the mitotic index was computed on the basis of counts performed on four fields in the outgrowth from each culture. In many cases these results were also checked by a statistical consideration of the spread of the mitotic indices calculated for each of the four fields. Only one example will be given here. When the data summarized in Table I are so examined, the mean value for the mitotic index with colchicine 10^{-5} is found as 0.159 with a standard error of 0.021; for colchicine 10^{-5} + *meso*-inositol 10^{-3} it is 0.050 ± 0.014. This difference is significant. With *meso*-inositol 10^{-3} alone the mitotic index is 0.017 ± 0.002 and in the saline control 0.017 ± 0.005. The difference between the results obtained with colchicine 10^{-5} + *meso*-inositol 10^{-3} and in the saline control is probably not significant.



Graph I. Colchicine and *meso*-inositol, effect on rat fibroblasts in vitro (3 experiments combined).



Graph II. Colchicine 10^{-5} molar + *meso*-inositol and related compounds 10^{-3} molar, at 20 hours.

TABLE I

Colchicine and *meso*-Inositol. Fixed after 20 hours.
(See Graphs I & II.)

Agent	Total No. Cells	Total No. Mitoses	Mitotic Index
Colchicine 10^{-5} molar	6 721	1 130	0.168
Colchicine 10^{-5} + *Meso*-Inositol 10^{-3} at same time	7 441	333	0.044
Meso-Inositol 10^{-3} molar	6 203	96	0.015
Control—saline	6 273	97	0.015

TABLE II

Meso-Inositol 10^{-3} alone for 24 hours. Then Colchicine 10^{-5} added.
Fixed after 20 hours.

Agent	Total No. Cells	Total No. Mitoses	Mitotic Index
Colchicine 10^{-5} molar 20 hours	6 446	1 018	0.158
Meso-Inositol 10^{-3} molar 44 hours + Colchicine 10^{-5} molar 20 hours	5 338	363	0.068
Control—saline	4 847	24	0.005

TABLE III

Meso-Inositol 10^{-2} 5 days. Then Colchicine $10^{-6} \times 5$ added. Fixed after 20 hours.

Agent	Total No. Cells	Total No. Mitoses	Mitotic Index
Colchicine $10^{-6} \times 5$ 20 hours	2 931	469	0.160
Meso-Inositol 10^{-2} 6 days + Colchicine $10^{-6} \times 5$ 20 hours	3 321	190	0.057
Meso-Inositol 10^{-2} 6 days	2 880	59	0.020
Control—saline	3 239	20	0.006

TABLE IV

Meso-Inositol 10^{-3} alone for 13 days. Then Colchicine $10^{-6} \times 5$ added. Fixed after 20 hours.

Agent	Total No. Cells	Total No. Mitoses	Mitotic Index
Colchicine $10^{-6} \times 5$ 20 hours	5 074	845	0.166
Meso-Inositol 10^{-3} 14 days + Colchicine $10^{-6} \times 5$ 20 hours	5 633	350	0.062
Meso-Inositol 10^{-3} alone 14 days	5 446	44	0.006
Control—saline	4 705	15	0.003

TABLE V

Meso-Inositol Compared with Related Compounds, No Colchicine. Fixed after 20 hours. (See Graph II.)

Agent	Total No. Cells	Total No. Mitoses	Mitotic Index
Meso-Inositol 10^{-3} molar	8 195	208	0.025
Dextro-Inositol 10^{-3}	8 290	173	0.020
Dextro-Sorbitol 10^{-3}	9 367	141	0.015
Control—saline	7 876	207	0.026

TABLE VI

Meso-Inositol 10^{-3} and Related Compounds + Colchicine 10^{-5}. Fixed after 20 hours. (See Graph II.)

Agent	Total No. Cells	Total No. Mitoses	Mitotic Index
Meso-Inositol 10^{-3} + Colchicine 10^{-5}	7 146	336	0.047
Dextro-Inositol 10^{-3} + Colchicine 10^{-5}	4 177	415	0.099
Dextro-Sorbitol 10^{-3} + Colchicine 10^{-5}	5 472	632	0.115
Control—saline + Colchicine 10^{-5}	4 469	498	0.111

TABLE VII

Meso-Inositol Compared with *Meso*-Inosose and *Epi*-Inosose. No Colchicine. Fixed after 20 hours. (See Graph II.)

Agent	Total No. Cells	Total No. Mitoses	Mitotic Index
Meso-Inositol 10^{-3} molar	5 635	106	0.018
Meso-Inosose 10^{-3}	5 157	63	0.012
Epi-Inosose 10^{-3}	4 842	64	0.013
Control—saline	5 219	59	0.011

TABLE VIII

Meso-Inositol, *Meso*-Inosose, *Epi*-Inosose, Effects on Colchicine action. Fixed after 20 hours. (See Graph II.)

Agent	Total No. Cells	Total No. Mitoses	Mitotic Index
Meso-Inositol 10^{-3} molar + Colchicine 10^{-5}	7 527	632	0.084
Meso-Inosose 10^{-3} + Colchicine 10^{-5}	5 487	961	0.175
Epi-Inosose 10^{-3} + Colchicine 10^{-5}	5 908	1 029	0.174
Control—saline + Colchicine 10^{-5}	5 459	865	0.158

TABLE IX

Colchicine, Sucrose and Ribose. Fixed after 20 hours. (See Graph II.)

Agent	Total No. Cells	Total No. Mitoses	Mitotic Index
Colchicine 10^{-5} molar, alone	8 649	2 218	0.256
Colchicine 10^{-5} + Sucrose 10^{-3}	6 900	2 007	0.290
Colchicine 10^{-5} + Ribose 10^{-3}	4 459	1 272	0.260
Control—saline	4 385	80	0.018

TABLE X

Colchicine and Glucose. Fixed after 20 hours.
(See Graph II.)

Agent	Total No. Cells	Total No. Mitoses	Mitotic Index
Colchicine 10^{-5} molar, alone	4 311	1 534	0.355
Colchicine 10^{-5} + Glucose 10^{-3}	4 280	1 452	0.339
Glucose 10^{-3} alone	2 966	81	0.027
Control—saline	4 378	130	0.029

TABLE XI

Colchicine 10^{-5} alone. Fixed after 4, 8, 12 and 16 hours.
(Figures for 20 and 44 hours added from other experiments.)

Agent: Cholchicine 10^{-5}	Total No. Cells	Total No. Mitoses	Mitotic Index
Fixed at 4 hours	3 123	22	0.007
Fixed at 8 hours	3 501	263	0.075
Fixed at 12 hours	3 542	430	0.121
Fixed at 16 hours	2 865	430	0.150
Fixed at 20 hours	6 721	1 130	0.168
Fixed at 44 hours	9 052	1 518	0.167

TABLE XII

Colchicine 10^{-5} + *Meso*-Inositol 10^{-3} at same time. Fixed after 4, 8, 12 and 16 hours.
(Figures for 20 and 44 hours added from other experiments.)

Agent: Colchicine 10^{-5} + *Meso*-Inositol 10^{-3}	Total No. Cells	Total No. Mitoses	Mitotic Index
Fixed at 4 hours	3 288	18	0.005
Fixed at 8 hours	3 566	245	0.068
Fixed at 12 hours	3 330	511	0.153
Fixed at 16 hours	3 394	259	0.076
Fixed at 20 hours	7 441	333	0.044
Fixed at 44 hours	9 862	407	0.042

Mitotic index of Saline or *Meso*-Inositol Controls is 0.007 at 20 hours.

DISCUSSION

The period between 12 and 16 hours would seem to be crucial for an analysis of the effect of *meso*-inositol in blocking the action of colchicine, and further study of this will be necessary. From the data on hand (averaged figures for all experiments) it seems that the percentage of prophases in the total mitotic counts of all colchicine groups is only one-third the percentage in colchicine + *meso*-inositol; and the percentage of anaphase and telophase (considered together) is a little less than one-third. The actual numbers are small, but they may be significant. The residue of divisions in metaphase is 97.9 per cent in colchicine alone and 93.7 per cent in colchicine + *meso*-inositol. If we accept the estimate of Hoffman (6) that a tissue mitosis takes from 30 to 40 minutes at 37°, these small actual differences in percentages at the moment of fixation may indicate a trend of some importance toward the drop in mitotic index that occurs in the presence of *meso*-inositol. In Love's tissue culture material (7), the interkinetic period was figured as 10 hours, with an average mitotic index of 0.015 at any given time.

In our controls the average figures are 20 per cent prophase, 36 per cent metaphase, 44 per cent anaphase and telophase combined. Though *meso*-inositol brings about a drop in mitotic index to one-fourth to one-half the index in colchicine alone, this lowered index still measures 2 to 7 times that of saline controls. Consequently there must be a large number of cells that have undergone a metaphase arrest that is not reversed by *meso*-inositol as long as colchicine is present. However, if the colchicine in 10^{-5} concentration is washed out after 20 hours, the metaphase arrest can be reversed by simple refeeding. It is hoped that study of cells fixed at intervals shorter than 4 hours, and cinephotomicrography, may further elucidate this problem. For extensive discussions of the duration of mitosis and of its phases, the reader is referred to W. von Möllendorf (10, 11, 12) and of phase distribution in the presence of colchicine *in vitro*, to O. Bucher (1). Friedmann *et al.* (5) discuss the reliability of phase distribution in an interesting analysis of the anti-mitotic action of certain quinones and their relation to the active principle of the colchicine group of poisons.

At 4 hours after treatment with colchicine, mitoses are present in all phases, though the percentages are skewed in favor of prophase and metaphase. Some nuclei are pyknotic. By 8 hours, 85 per cent of the mitoses are in metaphase, and the chromosomes though not actually clumped, lie close together at the equator. At 12 hours, the chromosomes seem to have lost their organization as a group and lie scattered; the percentage of metaphases

has increased to 90. At 16 hours, the cytoplasm appears to have decreased in viscosity, so that the cell surface is often iregular or pulled out in bizarre drop-like shapes; metaphase 96 per cent. The series treated with *meso*-inositol and colchicine together have essentially the same aspect in these 4 stages, except for the quantitative elements discussed above.

A high concentration of *meso*-inositol, such as 10^{-2}, seems to increase the general viscosity of the cells; but this concentration is at the borderline of cell injury, and it does not decrease the mitotic index any further than 10^{-3} does. Moreover, *d*-inositol and *D*-sorbitol induce the same cellular appearance, but do not decrease the index. For this latter reason, we cannot believe that *meso*-inositol merely interferes with the permeability properties of the cell membrane. Whatever is contributed by *meso*-inositol would seem to be of a specific nature; if colchicine is a spindle poison, possibly *meso*-inositol counteracts it by contributing to the formation of the spindle or by interfering with its prevention or dissolution by colchicine. Our data so far do not prove whether this influence of *meso*-inositol is exerted after the mitosis has begun or during the interkinetic period, though the phase percentages might seem to indicate the latter. [On this point the observations of Lüscher (8) on the Tubifex egg may be pertinent. He notes that colchicine brings about degeneration of the nuclear apparatus in interphase, so that the spindle cannot form; but if the spindle should be produced, the whole mitotic cycle proceeds, and the blocking takes place in the next resting stage.]

The experiments covering long-time exposure to *meso*-inositol indicate that the compound acquires no additional effect by being available for as long as 13 days. Nevertheless, when applied with colchicine it begins to exert its effect at the end of a period (12 hours) corresponding roughly to the length of the normal interkinetic period. This observation may have no significance, since there is technically no interkinetic period in cells which have undergone metaphase arrest. However if, as it appears, *meso*-inositol allows this arrest to be terminated after a duration *equivalent* to the interkinetic period, it might be speculated that *meso*-inositol replaces some necessary metabolite, which is affected by colchicine, and thus allows the cell to prepare for a new mitosis.

It is, of course, too early to speculate on the manner in which *meso*-inositol exerts its effect. That this effect may be considered as specific, is demonstrated by the failure of numerous control substances, studied in the course of this work, to act in a similar fashion. Sucrose, glucose, ribose, sorbitol were without effect. Even more remarkable is the ineffectiveness of *d*-inositol, *meso*-inosose and *epi*-inosose. The sterical relationships governing the bio-

logical oxidation of the various inositol isomers have been considered in a previous publication from this laboratory (9). The fact that not only one of the stereo-isomers of *meso*-inositol, but also two of its oxidation products were inactive makes it unlikely that the utilization of inositol by the cell proceeds *via* an oxidative mechanism. The general pathways through which *meso*-inositol is degraded or used by the mammalian organism are still unknown. In the particular case discussed here it would appear not improbable that inositol is first converted to a derivative, perhaps by partial phosphorylation of a lipid of the type of lipositol, and is utilized by the cell in this form.

SUMMARY

1. *Meso*-inositol exerts a specific effect in blocking the action of colchicine on dividing fibroblasts from the adult rat.

2. This effect of *meso*-inositol first becomes apparent (in the mitotic index) at about 12 hours after exposure, which corresponds roughly to the length of the interkinetic period.

3. It increases rapidly between 12 and 16 hours after exposure and is practically completed by 20 hours, at which time from 50 to 75 per cent of the metaphase arrests originally induced by colchicine have been reversed. The effect of *meso*-inositol is not increased by exposing the cells to it for as much as 13 days before the administration of colchicine.

4. The most effective combination of concentrations is 10^{-5} M colchicine and 10^{-3} M *meso*-inositol.

5. Neither *d*-inositol, *meso*-inosose, *epi*-inosose, *D*-sorbitol, ribose, sucrose, nor glucose, applied under parallel conditions, has any significant effect in blocking or reversing the action of colchicine.

REFERENCES

1. Bucher, O., *Z. Zellforsch. u. mikr. Anat.*, **29**, 283 (1939).
2. Carpentier, S., and Fromageot, C., *Biochim. et Biophys. Acta*, **5**, 290 (1950).
3. Chargaff, E., Stewart, R. N., and Magasanik, B., *Science*, **108**, 556 (1948).
4. D'Amato, F., *Caryologia*, **1**, 223, 358 (1949).
5. Friedmann, E., Marrian, D. H., and Simon-Reuss, I., *Brit. J. Pharmacol.* **3**, 263 (1948).
6. Hoffman, J. G., *Science*, **106**, 343 (1947).
7. Love, W. H., *Arch. exptl. Zellforsch. Gewebezücht.*, **10**, 442 (1930).
8. Lüscher, M., *Rev. Suisse Zool.*, **52**, 349 (1945).
9. Magasanik, B., and Chargaff, E., *J. Biol. Chem.*, **174**, 173 (1948).
10. von Möllendorff, W., *Z. Zellforsch. u. mikroskop. Anat.*, **27**, 301 (1937).
11. —— *Arch. exptl. Zellforsch. Gewebezücht.*, **21**, 1 (1938).
12. —— *Z. Zellforsch. u. mikroskop. Anat.*, **28**, 512 (1938).
13. Simms, H. S., and Sanders, M., *Arch. Path.*, **33**, 619 (1942).

NUCLEAR INCORPORATION OF P^{32} AS DEMONSTRATED BY AUTORADIOGRAPHS

ALMA HOWARD and S. R. PELC

Medical Research Council, Radiotherapeutic Research Unit, London, Great Britain

Received August 2, 1950

INTRODUCTORY

It has been shown (3) that cells can be labelled with radioactive phosphorus, i.e. that at least some of the P^{32} taken up and synthesized into organic compounds insoluble in acetic-acid alcohol and water remains in the cell and passes to its daughter cells, and that the presence of P^{32} in individual cells can be detected by the autoradiograph method. It has recently been observed (4) that tissue cultures of chick fibroblasts grown for 46 hours in media containing P^{32}, then fixed in 80 per cent alcohol and subsequently extracted with normal HCl at 60° C, contain some resting nuclei which show definite autoradiograph, while others show none or only very weak autoradiograph. There are three possible explanations of this: (a) P^{32} in the form of an organic compound which withstands fixation and hydrolysis is incorporated during part of the cell cycle; (b) that it is incorporated only in some cells; (c) that phosphorus is lost from some cells, but not from others, either during life or in the histological manipulation of the tissue. The last possibility seemed to us the least likely.

A material in which the number of cells showing autoradiograph can be related to the number of dividing and differentiating cells should be suitable for a study of this uptake of phosphorus.

TECHNIQUE

For this investigation roots of *Vicia faba* were used in which the rapidly growing meristem occupies a well defined portion of the tip while the elongating cells above show very few divisions. Following the method described by Gray and Scholes (2), seedlings were cultured at 19° C and the length of the primary root measured. Groups of 4 roots were immersed in tap water in tubes of 100 ml capacity through which air was constantly bubbling. P^{32} in the form of NaH_2PO_4 with 16 μg/ml of carrier

was added. Treatment was for 2, 6, 24 and 48 hours, the dosage being 1 μC/ml for half of the 2 and 6 hour samples and 0.2 μC/ml for the other half of the 2 and 6 hour samples and for all the 24 and 48 hour samples. Over a period of 12 days there was no difference in the growth rate of control roots and roots grown for 48 hours in 0.2 μC/ml. Radiation damage can therefore be neglected at the lower concentrations of P^{32} and probably also at the higher ones in view of the much shorter exposures and lower total dose delivered. Immediately after treatment the roots were cut off and fixed in acetic-acid alcohol (1 : 3) for 1 hour, washed in water for 1 hour, hydrolysed at 60° C in normal HCl for 10 minutes, washed for 1 hour and, after removal of the root cap, cut into segments 2 mm long. These segments were divided into four sectors and squashed separately in 45 per cent acetic acid, the cover slips floated off in 10 per cent acetic acid, and the slides, after washing for at least 1 hour in distilled water, autoradiographed by the stripping film method (1). Preparations were exposed for periods which varied from 3 31 days. Other roots, after the same treatment and fixation, were sectioned longitudinally at 10 μ, hydrolysed as above and autoradiographed. After photographic processing, slides were mounted in a chrome gelatin mixture, which preserves both film and tissue in good condition.

RESULTS

Most observations were made on unstained material by phase contrast microscopy, which gives excellent images of cell and nuclear structure. (Fig. 1.) Staining by the Feulgen method before application of the photographic film gives good results; this stain is not affected by photographic processing (Fig. 2). Preparations of control roots without P^{32} showed no correlation between background grains and cells and were free from artefacts.

In squashes some cells show clear individual autoradiographs, which consist generally of a definite concentration of grains over the resting cell nucleus (Fig. 1). The density distribution of the grains exhibits features to be expected if they are the result of beta-particles from P^{32} in the nucleus. In dividing cells in the 24 or 48 hour samples the grains are concentrated over the chromosomes (Fig. 2). The resolving power of the autoradiographs is 2 μ or slightly better.

Some cells show autoradiograph over the cytoplasm, and in these cases the grains frequently show small concentrations unrelated to the nucleus and are not distributed at random over the cytoplasmic area. Another pattern of autoradiograph frequently encountered is a concentration over the area of the developing cell wall at late telophase; this autoradiograph is usually well separated from the autoradiographs due to the two daughter nuclei. These non-nuclear sites of P^{32} are both interesting and significant, but are outside the scope of this paper and after further investigation will be described and discussed in another paper.

The statistical distribution of cells whose nuclei show autoradiograph, in relation to time of treatment and to linear distance from the tip of the root, is shown in Table I. The segment 0–2 mm from the tip includes most of the meristem: above this level the cells elongate and division is rare in the cortex. From measurements of linear growth during the treatment period of the root concerned, the segments in the upper parts of the roots were selected as having been above the level of the meristem at the time when treatment with P^{32} began.

The Table shows that there are few 'positive' cells during the first two hours. After 6 hours 39 per cent of resting nuclei in the meristem show autoradiograph: thus while there has been time for incorporation of P^{32}, this has occurred in only some of the cells. None of the cells in process of division in the 6 hour roots show autoradiograph. This indicates that incorporation of P^{32} takes place during the resting stage and not during division. Further, the fact that only some of the resting cells show autoradiographs after 6 hours suggests that this incorporation occurs during only a part of the resting stage.

The number of cells showing autoradiograph in the upper segment of the root is in every case very much less than the number in the meristem. The figure goes up from 3 per cent after 6 hours to 22 per cent after 48 hours. These cells were already elongating when treatment began, and the number which divided since P^{32} became available to them is small. Further investigation of this point cannot conveniently be made on squashes since the normal arrangement of the cells is destroyed. The resolving power of autoradiographs is, however, better in squashes than in sections because single layers of whole cells are obtained. Sections have the advantage of retaining the arrangement of the tissue and therefore offer means of comparing cells in different parts of the root.

In sections, autoradiograph appears above the meristem in all samples except the 2 hour one. The elongating cells of the cortex show autoradiograph near the meristem in 24 and 48 hour roots, but the number of nuclei showing autoradiograph diminishes with increasing distance from the meristem. Over the nuclei of the developing vascular system autoradiograph appears in 6, 24 and 48 hour samples as far up the root as sections were cut, i.e. 5 cms. It should be emphasized that this autoradiograph is above the nuclei, and that from the method of preparation it cannot be due to water soluble phosphorus. Fig. 3 shows the percentage of cortical cells showing autoradiograph over the nucleus as a function of the distance from the root tip for roots which grew 3 cms in 24 hours (x) and 4.2 cms in 48 hours (O)

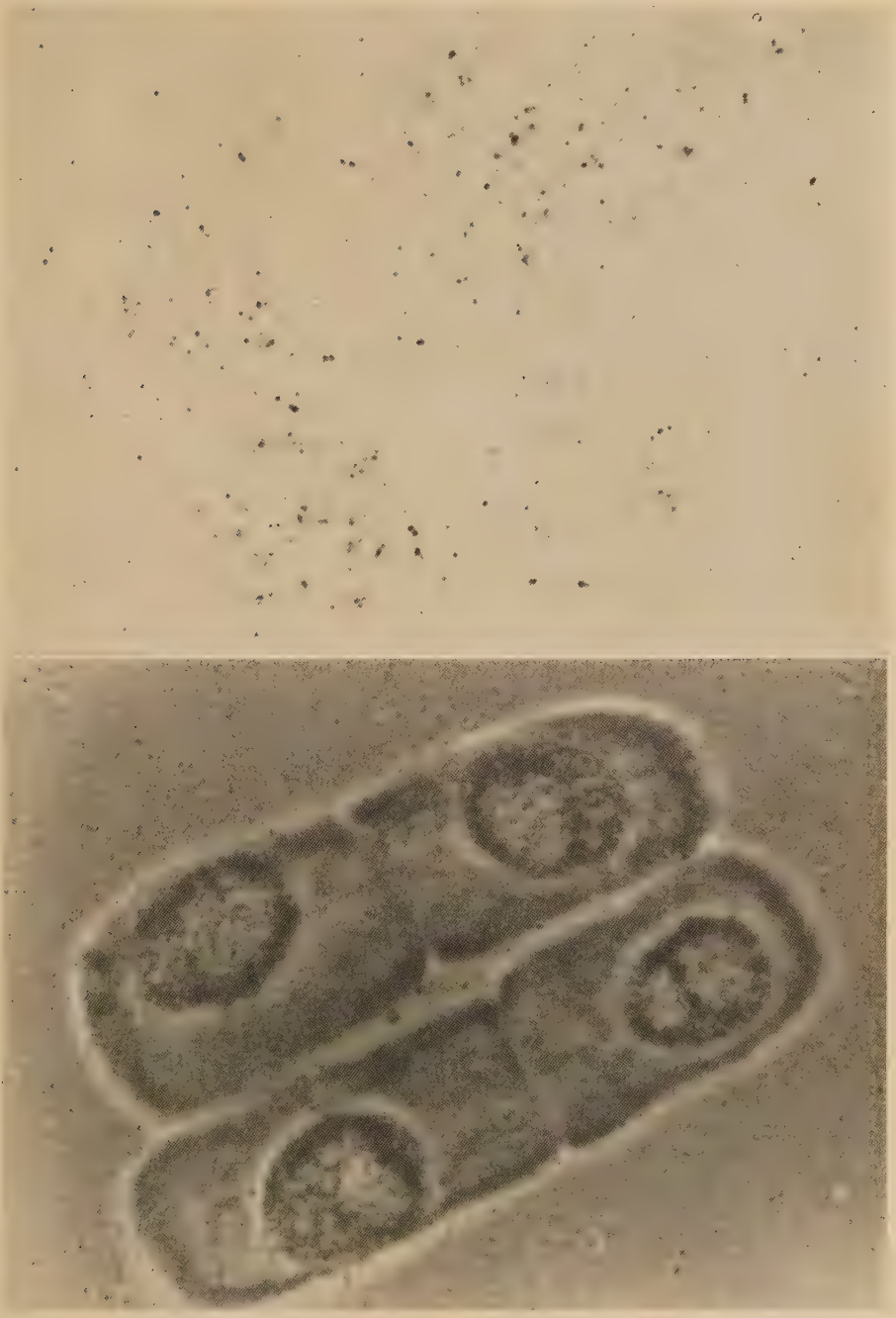


Fig. 1. 4 resting cells, *Vicia faba*, root meristem, 3 nuclei showing autoradiograph. 6 hours P^{32} (1 μC/ml) 30 days exposure. a) phase contrast, b) same field without phase contrast. × 1600.

Fig. 2. Early anaphase, *Vicia faba*, root meristem, Feulgen staining. 40 hours P^{32} (1 μC/ml), 14 days exposure. × 1600.
a) focussed on the chromosomes.
b) focussed on the photographic grains.

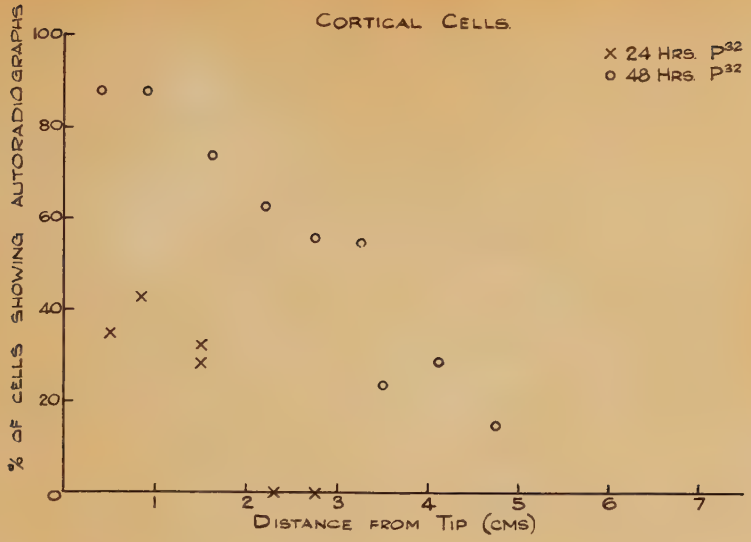


Fig. 3. Percentage of cortical cells showing autoradiograph as a function of distance from the root tip.

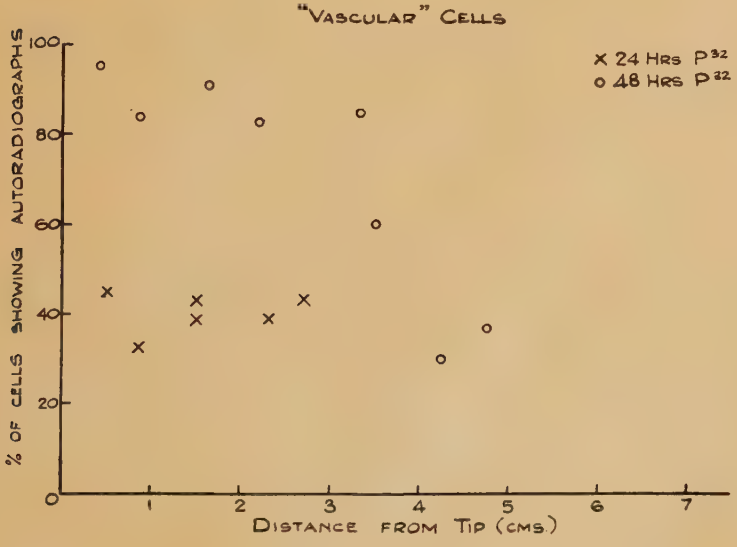


Fig. 4. As Fig. 3, but for cells of the developing vascular system.

respectively. Each curve starts at a point corresponding to that found in the meristem in squashes, and then falls to zero with increasing distance from the tip. The initial high value for the percentage is explained by the fact that the elongating cells just above the meristem are the daughter cells of divisions which have occurred in the meristem during the whole period of P^{32} treatment. As the distance from the root tip increases cells are encountered which began differentiation at times shorter than the full period of P^{32} treatment. Fig. 3 shows that a value of about 40 per cent is found near and in the meristem after 24 hours. This value is found at a distance of 2 ½–3 cms from the tip in the 48 hour root, which agrees well with the increment in length of this root during the second 24 hour period. Fig. 4 illustrates the percentage of cells showing autoradiograph in the developing vascular system. In the 24 hour root the sections were too short to follow the vascular system above 3 cms; in the 48 hour root the percentage of cells decreases with a slope similar to that seen for the cortical cells (Fig. 3), but the decrease begins at about 3 cms from the tip. Since cell division occurs in the developing vascular tissue, and furthermore, this is the tissue which would have given rise to lateral roots, it is clear that at least some of these cells retain the capacity for division. The relatively high percentage of autoradiographs in the top segments in Table I are therefore at least partly due to vascular cells.

The sectioned material also showed isolated regions of very heavy autoradiograph around the edge and at especially close intervals around the root cap. These autoradiographs are frequently associated with material of some sort, but its nature could not be identified; it does not have the appearance of a fungus, though this possibility cannot be excluded at this stage.

DISCUSSION

It would seem that radioactive phosphorus has been incorporated in a stable form by synthesis and not by exchange for inactive phosphorus atoms in already synthesized compounds. Exchange processes appear to be negligible for our purposes because a considerable number of resting nuclei show no autoradiograph, while others on the same slide show definite autoradiograph. Replacement of stable phosphorus by P^{32} should be especially apparent in the older cells, at some distance from the tip, to which P^{32} was available during the whole period of its application. In fact, the proportion of cells showing autoradiograph decreases with distance from the tip (Figs. 3 and 4).

TABLE I

Bean Root Squashes.
2 hr. and 6 hr. beans in 1.0 μC P^{32}/ml.
24 hr. and 48 hr. beans in 0.2 μC P^{32}/ml.

Hours in P^{32}	Distance from the root tip (millimetres)		Per cent of cells showing autoradiograph		Cells counted	
2	0–2		1	—	70	Only 1 cell showing very weak autoradiograph.
2		10–12	—	4	51	
6	0–2		39	—	69	Dividing cells show no autoradiograph.
6		14–16	—	3	33	
24	0–2		48	—	111	Dividing cells show autoradiograph.
24		35–37	—	12	33	2 of the 4 cells showing autoradiograph are prophases.
48	0–2		94	—	63	
48		50–52	—	22	31	

Evidence that only resting cells which are preparing for division incorporate P^{32} in the chemical compounds observed is provided by the fact that the percentage of nuclei showing autoradiograph decreases with the distance from the root tip in the differentiating cells.

Phosphorus is incorporated during a part of the resting stage and not during mitosis. If phosphorus were taken up during the whole of the resting stage, all resting nuclei would be expected to show autoradiograph, which should merely increase in density during the time P^{32} is available; in fact, only some of the resting cells show autoradiographs at 6 hours. This, and the fact that no nucleus in division, not even those in early prophase, showed an autoradiograph at 6 hours, but all showed it at 24 hours seems to indicate that the incorporation of phosphorus stops some time before visible prophase, and that no synthesis takes place during the actual division.

Gray and Scholes (2) considered the equilibrium between meristematic and differentiating cells. Their calculations lead to an estimate of an average time of 20 hours for interphase and 2 ½ hours for the actual division. Thus one half of the cells present in the meristem at any given time will undergo

division within 22 ½ hours and one half will differentiate. Our view that the presence of an autoradiograph over the nucleus in our observations indicates preparation for division would lead us to expect autoradiograph in about 50 per cent of the cells in the 24 hour sample, which agrees with the observations. (48 per cent in Table I, see also Fig. 3.) During 6 hours about one quarter of the potentially dividing cells should undergo division. If no P^{32} is taken up during the 2 ½ hour period of division, nor for a comparable time before division, then the number showing autoradiograph at 6 hours should be about one quarter less than that in the 24 hour sample, i.e. about 37.5 per cent (39 per cent in Table I). It thus appears that the 'inactive' period before visible prophase begins is of the order of 2 hours as assumed, but the accuracy of our measurements is too low to place great reliance on this estimate. For a more accurate calculation various factors such as the delay period before autoradiographs can be assessed with certainty and the lower limits of their observability would have to be taken into account. After 48 hours all cells preparing for differentiation during the first 24 hours will be outside the meristem and practically all cells in the meristem will be daughters of cells which have divided during the first 22 ½ hours. Furthermore, one half will have undergone a second division during the second 24 hour period or will have prepared for it. Thus all cells should show autoradiograph (94 per cent in Table I).

These experiments show that the presence of P^{32} in cells can be detected with a resolving power of approximately 2 μ, and demonstrate the possibility of using autoradiographs as a cytochemical method, with the additional advantage of being able to determine the time of uptake. An estimate of the duration of the mitotic cycle could be gained from detailed analyses of more closely spaced experiments. Finally, the method promises to be useful where an estimate is required of the number of potentially dividing cells in a tissue.

The phosphorus compounds whose phosphorus is not dissolved during the handling and preparation of the tissue are likely to be nucleic acids, phosphoproteins and phospholipids, but it must not be inferred that other compounds may not be present. It seems likely that the autoradiographs obtained are mainly due to P^{32} in the form of nucleic acids; in the nuclei it is probably desoxyribonucleic acid.[1] Although the spatial and chemical distribution of phosphorus in the cell is of great general interest, the exact

[1] Recent experiments have shown that desoxyribonuclease removes the phosphorus compound which gives rise to the nuclear autoradiographs described in this paper. We therefore conclude that they are due to P^{32} incorporated in desoxyribonucleic acid which was synthesised since P^{32} was made available to the roots.

form in which the element is present in this instance is irrelevant to our argument since the object of the investigation was to determine whether phosphorus is incorporated in a bound form during all stages or only certain stages of cell growth and whether its incorporation is related to the subsequent mitotic behaviour of the cell. The timing of the incorporation of nucleic acids in relation to the mitotic cycle has been a matter of controversy for some time; it would be inappropriate to discuss this aspect of our observations until more information regarding the chemical form of the phosphorus compounds which we observe is available.

SUMMARY

Roots of *Vicia faba* seedlings were grown for periods of 2 to 48 hours in solutions of P^{32} in the form of NaH_2PO_4. Stripping-film autoradiographs were made of squashes and sectioned material from the meristem and from proximal segments where cell divison is rare. It is concluded (a) that P^{32} enters into organically bound form (probably desoxyribonucleic acid) in the nucleus during the resting stage, but not during the actual division of the cell; (b) that P^{32} is incorporated in this form only in nuclei which are preparing for division; (c) that P^{32} in this form is inherited by the daughter cells.

REFERENCES

1. Doniach, I., and Pelc, S. R., *Brit. J. Radiol.*, **23**, 184 (1950).
2. Gray, L. H., and Scholes, M. E., *Brit. J. Radiol.* **24**, 82 (1950).
3. Howard, Alma, and Pelc, S. R., *Brit. J. Radiol.* **23**, 634 (1950).
4. Pelc, S. R., and Spear, F. G., unpublished (1950).

FURTHER OBSERVATIONS ON BIOLOGICAL REACTIONS BY THE CARTESIAN DIVER TECHNIQUE — WITH SPECIAL REFERENCE TO MAMMALIAN TISSUES

A. BORROW and J. R. PENNEY

Human Nutrition Research Unit, Medical Research Council Laboratories, London

Received August 13, 1950

INTRODUCTION

Previous work using the Cartesian Diver has been mainly done by the Carlsberg group, in their studies on the respiration of Amoebae; and by Boell, Needham, and co-workers (3, 4, 5, 6, 18) who determined the respiration, glycolysis and R.Q. of embryonic tissue. Linderstrøm-Lang and Glick (16) used the technique for the determination of cholinesterase in horse serum; and Anfinsen (2) for the estimation of diphosphopyridine nucleotide in layers of bovine retina. In the present communication these writers wish to report progress made in the preparation and handling of mammalian tissues for study by the diver technique. Rat diaphragm, and the muscular coat of the rat intestine have been used as examples of "intact material" for measurement of oxygen uptake, respiratory quotient and aerobic glycolysis; the succinoxidase and transaminase enzyme systems have been examined, in liver homogenates. The systems reported illustrate the various types of mixing in the diver.

CARTESIAN DIVER TECHNIQUE

The apparatus used was the standard Cartesian Diver microrespirometer described by Holter (14) and the symbols are those used by this author; the nomenclature is given in Figure 1. The divers used throughout were coated with the water repellent silicone DC 200 (20, 22); the dimensions and fillings are given in the appropriate sections. Changes in gas volume were calculated according to the equations given by Holter (14).

With CO_2 it was assumed that the oil seal was fully saturated at the partial pressure of the CO_2 in the main gas-phase (V–ω). $\lambda_0 = 0.9$ and 0.57 at 37° for P-K and MOBA respectively).

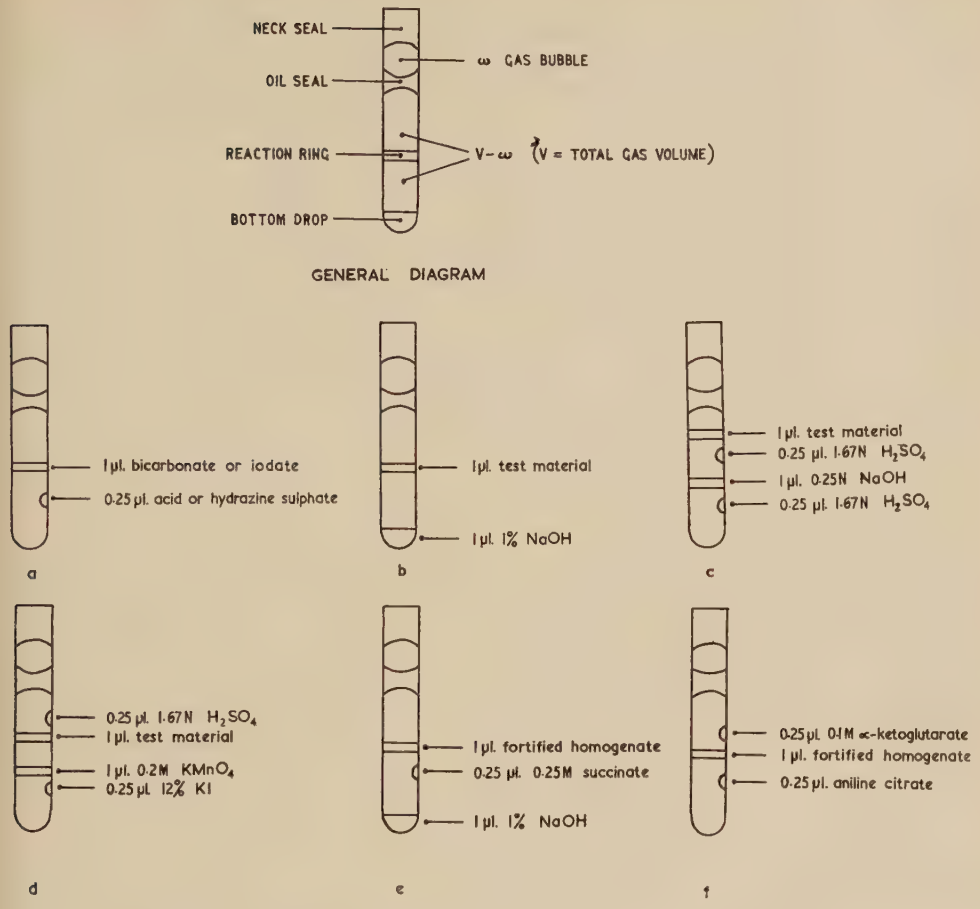


Fig. 1. Arrangement of reagents in Cartesian Diver.

Temperature

Preliminary investigations of the performance of the apparatus have been made at 37°, the temperature at which mammalian tissues are generally studied, since Linderstrøm-Lang (15) has suggested that additional errors might arise at this temperature. Also Boell and Nicholas (7) encountered difficulties when using paraffin-wax coated divers at 37°.

Air-filled divers of volume about 10 µl., and containing a bottom-drop of distilled water, the conventional paraffin-kerosene oil seal (P-K) and Holter medium neck seal were found to remain constant at their equilibrium pressures for at least eight hours. No difficulty was encountered in the adjustment of these divers to the equilibrium position, and consecutive readings taken at the equilibrium pressure were found to be as constant as at 30° C.

Table I

	Temp.	Nature of seal	Range µl	No. of Observations	Mean recovery per cent	S.E. of per cent deviations
N_2	30° C	P-K	0.25–0.70	20	99.45	1.42
	37° C	P-K	0.02–0.40	16	100.75	2.15
			0.11–0.40	12	100.15	1.61
		MOBA	0.05–0.45	16	100.20	2.90
CO_2	30° C	P-K	0.12–1.13	18	99.5	1.14
	37° C	P-K	0.02–1.10	14	99.4	2.33
			0.13–1.10	10	100.2	1.67
		MOBA	0.05–1.10	17	99.2	1.63
			0.13–1.10	13	99.5	1.51

CO_2 and N_2 evolutions were determined as described by Waterlow and Borrow (22), the diver filling being shown in Fig. 1 a, and the results compared with those obtained by these workers at 30° (Table I). It will be seen that the recoveries and deviations obtained at the two temperatures over comparable ranges are similar. Since the absolute error involved in the technique is constant, the deviation is larger in the lower range. All the experiments reported below were carried out at 37° C., and no difficulties which could be attributed to the higher temperature were encountered.

Carbon Dioxide Leakage

Loss of CO_2 by leakage does not give rise to significant error when the experimental period is very short, e.g., in the liberation of CO_2 by acid; but errors do arise during the continuous evolution of CO_2 or in experiments which employ CO_2 in the gas-phase. Four methods for reducing this loss have been discussed by Linderstrøm-Lang and Holter (17); reduction of the cross section of the diver neck, increase in the length of the mouth seal, saturation of the flotation medium with the same gas-mixture as is employed in the diver, and the use of loosely fitting glass stoppers in either the medium or oil seal to reduce the leakage of CO_2 through it. Precautions involving these methods either singly (6, 17) or in combination (22) have been taken but when large differences of CO_2 concentration in the gas-phase occur in the experiment, these precautions are inadequate. Such a condition obtains in the second method of Dickens and Šimer (10) for the determination of R.Q's., where all the CO_2 in the gas-phase is finally absorbed; diffusion of CO_2 into the diver from the medium saturated with CO_2 prevents the attainment of

equilibrium conditions. Accordingly an attempt has been made to reduce the loss of CO_2 from the diver by modification of the oil seal.

In our experience, no advantage is gained by replacing paraffin oil by mineral oil alone, a finding previously reported by Holter (14). Since the addition of solutes to solvents is known to decrease gas solubilities, an effect well illustrated by comparing the solubility of CO_2 in water and in Holter medium, an attempt was made to apply this principle. The effect on CO_2 leakage of several acidic organic compounds dissolved in mineral oil has been determined. Benzoic acid, found to be the most effective, reduced the CO_2 leakage to about 20 per cent of that value found with the conventional paraffin oil seal. A mineral oil, Carnea 21[1], saturated with benzoic acid at 37° C., pipetted easily and the benzoic acid did not crystallise out within one hour of removing the mineral-oil-benzoic-acid solution (MOBA) from 37° C., to room temperature.

Performance of Mineral-Oil-Benzoic-Acid Seal Compared with Paraffin Seal

The behaviour of air-filled divers containing a bottom-drop of distilled water, a seal of 1 μl of either P-K or MOBA, and the conventional medium neck seal was first examined. No significant change in the equilibrium pressure was observed in 18 hours. These divers were also subjected to pressure changes far in excess of those required in normal diver operations. The MOBA seal was found to be quite stable, and with both seals there was a return to the initial equilibrium position within 6 minutes.

The recoveries of N_2 and CO_2 were determined, and the results compared with those obtained using the P-K seal. The maximum reading was always attained within 10 minutes of the time of mixing. The results in Table I show that both oil seals give similar values for the means and the deviations.

Two methods were employed when comparing the CO_2 leakage rates from divers with P-K or MOBA seals; CO_2 was introduced into the diver either initially in a gas-mixture or in known varying amounts by liberation as above. 1 μl oil seals, and mouth seals 2.5–3.0 mm long were used. (Diver neck cross section 1.0–1.3 mm^2). Since the results obtained with both these methods were similar, they were averaged in Figure 2. The stability of air-filled divers and divers in which N_2 had been liberated is also shown. During the first hour of the experiments, the CO_2 loss was 4 $\frac{3}{4}$ per cent and about $\frac{3}{4}$ per cent with the P-K and MOBA seals respectively. The non-linearity of

[1] Obtainable from Shell Oil Co.

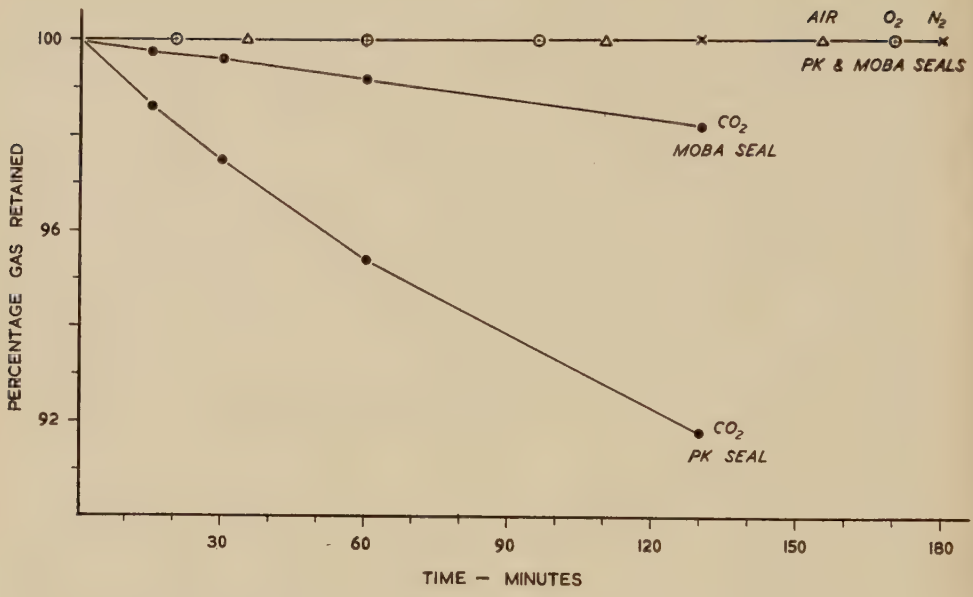


Fig. 2. Effect of P-K and MOBA seals on gas retention.

the P-K curve in the initial stages has been reported previously, using the lower temperatures (15, 22), but the deviation from linearity is less marked at 37° C. Since the greater rate of loss in the initial stages is due to increasing amounts of CO_2 being dissolved in the medium seal the lower solubility of CO_2 in Holter's medium at 37° C, probably accounts for the small deviation observed.

Finally, it was found that there was no significant loss of gas from divers containing 95 per cent O_2: 5 per cent N_2 and there was no significant increase in the length of the ω bubble. The oil seal would, therefore, appear to be a reasonable barrier to the diffusion of O_2 (15).

From the results of these experiments it appeared that the MOBA seal could effectively replace the P-K seal and in experiments involving the continuous evolution of CO_2 in the presence of CO_2 in the gas-phase it should reduce the error due to CO_2 leakage.

It has been shown that in the determination of plasma cholinesterase, comparable results are obtained by both the Warburg and Diver techniques provided loosely stoppered divers are used in conjunction with a flotation medium saturated with the same CO_2 gas-mixture as constitutes the initial gas-phase (22). Results obtained with such divers have been compared with those using unstoppered divers containing the MOBA seals and using the conventional Holter medium.

TABLE II

Cholinesterase. µl CO_2/ml plasma/min.

P-K and stopper	1st Method MOBA	2nd Method MOBA	2nd Method MOBA
Plasma dil. 10	10	10	50
47.0	46.7	46.3	47.3
48.0	47.0	46.6	45.4
46.7	46.7	47.3	43.8
46.7	46.7	46.6	45.4
47.0	47.0	47 3	45.4
46.7	46.7	46.6	45.4
47.0	46.8	46.8	45.45

In the first series of determinations the method was used as described by the above authors, the acetylcholine substrate being prepared in water. It will be seen from Table II that the results obtained by both methods are in good agreement. The blank determination for the CO_2 liberated from acetylcholine in bicarbonate will include a correction for leakage of initial gas-phase CO_2; nevertheless, the blank corresponded to 3–4 per cent of the CO_2 liberated in the experimental period, whichever method of leakage reduction was employed. This, together with the fact that the corrected results are in good agreement shows that the MOBA is at least as efficient in minimising leakage as the use of stoppers and gas-saturated medium.

In the second series of experiments, the substrate was prepared in bicarbonate solution as described by Linderstrøm-Lang and Glick (16) thus obviating the need for the blank determination. Two samples of the plasma previously employed, diluted ten and fifty times respectively, were used to show the effect of the absolute amount of CO_2 evolved in the course of the determination. The results are given in Table II.

With the plasma ten times diluted (actual CO_2 evolution about 0.15 µl), no difference could be detected between the results obtained and those in the first series of determinations. In the case of the plasma fifty times diluted (actual CO_2 evolution about 0.03 µl.), the final results were 3 per cent low.

In these experiments the amount of CO_2 initially present in all the divers was about 0.3 µl. Hence if the standard CO_2 leakage rate found previously is applied, i.e. $\frac{3}{4}$ per cent of the gas-phase CO_2 per hour, the loss of initial gas-phase CO_2 to be expected in 30 minutes is about 0.001 µl.; since the experimental period is extremely short the loss from evolved CO_2 can be neg-

lected. Thus the errors to be expected when the CO_2 evolved is 0.15 μl. and 0.03 μl., are < 1 per cent and 3 per cent respectively. This is in agreement with the experimental results.

DETERMINATION OF OXYGEN UPTAKE AND RESPIRATORY QUOTIENT

Preliminary Experiments with Yeast

For the determination of O_2 uptake, the method used by Holter (14) was employed with a slight modification of the diver filling. The respiring material is placed in a ring of buffer in the diver neck instead of in a bottom-drop (Fig. 1 b). This arrangement increases the rate of gas uptake permissible before the diffusion of gases through the buffer becomes limiting (15). Since the measurement of O_2 uptake is inherent in the determination of R.Q., it will not be dealt with here in any further detail.

Two principal methods are available for the determination of R.Q. in the Warburg respirometer, i.e. the first and second methods of Dickens and Šimer (9, 10). In order to study the adaptation of these methods to the Diver, yeast was selected as the experimental material since it provides an homogeneous suspension with a respiratory quotient of unity.

Adaptation of the First Method

Divers of neck area 1.0–1.3 mm^2 and volume about 10 μl were used with air as the gas-phase. The divers were charged as in Fig. 1 c, a yeast suspension in Ringer-Locke medium being the test material. Test divers and controls were in duplicate. Each diver was placed in its flotation vessel immediately the filling was completed. The beginning of the equilibration period was taken as the time at which the oil seal was placed in position. After 20 minutes equilibration, the base-line readings of all divers were recorded. Acid drop and reaction ring in the controls were then mixed by the application of a single large positive pressure. After 30 or 60 minutes' respiration the test divers were read and mixed in the same way; liberation of CO_2 was complete in 10 minutes. As in the Warburg method, the decrease in gas volume during the test period represents O_2 absorbed, and the CO_2 liberated on acidification, when corrected for the control, represents the respired CO_2. Some typical results are tabulated in Table III.

The above method differs from that of Boell *et al.* (3) in three ways. Firstly, smaller divers were used which apart from increased sensitivity are in our experience more stable at the equilibrium position; secondly, the test material was placed as a ring instead of a bottom-drop for the reasons given pre-

viously; lastly, we have found no need for their "piston-drop;" this may be due to the fact that we coat our divers with the silicone DC 200 instead of paraffin wax.

Adaptation of the Second Method

The chief obstacle in the adaptation of this method arose from CO_2 leakage. In early attempts, before the development of the MOBA seal, an effort was made to correct for such leakage but these corrections only proved applicable when the experimental period was short. The introduction of the MOBA seal has rendered such detailed correction unnecessary, but the method proved reliable within certain limits. For the purpose of comparison both methods are described.

Apart from the difference in the oil seals, the diver fillings were identical in both procedures. Divers of neck area 1.0–1.3 mm^2 and of volume 15 µl, were filled with 3 per cent CO_2 in O_2. The reagents, previously saturated with this gas-mixture and a yeast suspension in Ringer-bicarbonate solution were added, as shown in Fig. 1 d, with the diver under water (22). In order to ensure that the gas-volume below the oil seal ($V-\omega$) is constant, the position of the oil seal must be adjusted carefully. This (1 µl.) was first placed near to the mouth of the diver and then adjusted by means of a braking pipette (14) until the distance of the lower meniscus of the oil seal from the mouth of the diver was equal to a predetermined number of divisions of the eye-piece scale of a low-power microscope. The diver was then removed from the water so that the gasspace above the oil seal was open to the air, and the neck seal (2.3 mm) finally placed as usual.

After an equilibration period of 20 minutes, control and test divers were read and the reaction ring in the control diver immediately mixed with acid by the application of negative pressure. Equilibrium conditions were attained in 10 minutes, and after noting the manometer reading a positive pressure was applied to mix permanganate with iodide. All the CO_2 present in the gas-phase was absorbed by the resulting alkaline solution and a steady state was reached after 40–50 minutes. The same procedure was followed in the test divers after 30 minutes' respiration.

Determination of Initial Gas-phase CO_2 in the diver and Calcutation of Results

(1) USING P-K OIL-SEAL AND CORRECTING FOR CO_2 LEAKAGE

In the second method of Dickens and Šimer (10), the algebraic sum of the O_2 uptake and of the respired CO_2 is obtained from the gas volume changes which occur during the 30 minutes respiration period. Erroneously high values for O_2 uptake will be ob-

TABLE III

Respiratory Quotient of Yeast Suspensions.
(Arbitrary suspension; actual uptake figures. in μl.)

Method	CO_2	O_2	R.Q.
1st Method	0.154	0.156	1.01
		0.153	0.99
	0.019	0.020	1.06
		0.018	0.95
2nd Method	0.100	0.102	0.98
P-K correcting	0.101	0.104	0.97
for leak.	0.120	0.122	0.98
	0.122	0.122	1.00
2nd Method	0.160	0.162	0.99
MOBA seal	0.159	0.160	0.99
	0.086	0.088	0.98
	0.082	0.084	0.98
	0.117	0.119	0.98
	½ dil. 0.056	0.057	0.98

tained if loss of CO_2 occurs during this time. In order to obtain the respired CO_2 the volumes of initial CO_2 in the gas-phase and arising from the bound CO_2 of the buffer and the respiring material are subtracted from the total CO_2 present. If the original CO_2 figure for the initial CO_2 gas-mixture is taken, then the estimate of the respired CO_2 derived from this will be too low. The calculated amount of CO_2 taken up by the tissue will be further increased. Thus, although the values for the O_2 consumed and CO_2 respired might not be greatly in error, the value obtained from the R.Q. would be considerably affected. In the above, only leakage from the gas-phase of CO_2 initially present has been considered. In most of the experiments this is the main source of error, but by the appropriate calibration of the test and control divers used in the R.Q. determinations, the error can be reduced.

All divers were filled with the gas-mixture, and charged as described on page 194, except that the reaction ring consisted of water. In divers to be used for control the CO_2 in the gas-phase was determined 10 minutes after the equilibration period, to give the actual CO_2 content due to the gas-mixture in the diver at the beginning of the control absorption period. The loss of CO_2 from the divers to be used for test purposes was followed for the duration of the experimental period and the CO_2 content was found by absorption after a further 10 minutes, i.e. 40 minutes after the usual equilibration period. These calibrations were carried out in triplicate, and the in-

TABLE IV

Specimen protocol for Experiment on Yeast R.Q. determination using second method. P-K. correcting for leak.

CO_2 in gas-phase (previously determined)	0.193 µl
Initial CO_2 (tissue and medium; from control)	0.290 µl
Final CO_2 (tissue and medium)	0.292 µl
DV in reaction period	0.014 µl
CO_2 leak in reaction period (previously determined)	0.011 µl
Total CO_2 absorbed	0.586 µl
CO_2 respired	0.101 µl
O_2 respired	0.104 µl
R.Q.	0.97

dividual results did not vary more than ½ per cent of the mean, provided the time intervals were strictly adhered to. The mean values obtained were then used to correct for leakage in the calculation of the results as shown in Table IV. Some typical results are given in Table III.

(2) USING MOBA SEAL:

The results obtained by this technique, calculated by the method of Dickens and Šimer (10) are given in Table III. In order to obtain the CO_2 content of the gas-mixture, absorption of gas-phase CO_2 was effected at times corresponding to the absorption in control and test divers.

It will be seen that the values for R.Q. are only slightly less than unity. In these experiments, O_2 uptake was about 0.1 µl./30 minutes and the loss due to CO_2 leakage would be about 0.001 µl. Thus only a very small error is introduced, which will increase if the O_2 uptake is reduced or if the CO_2 content of the gas-mixture is increased. The application of a correction for the initial CO_2 leakage will then reduce the error obtained. For most practical purposes the accuracy of the diver method for measuring R.Q. compares very favourably with that of the Warburg technique.

APPLICATION OF THE DIVER METHODS TO THE DETERMINATION OF O_2 UPTAKE, RESPIRATORY QUOTIENT, AND GLYCOLYSIS OF MAMMALIAN TISSUE

The tissues chosen to investigate these techniques with mammalian tissues were the diaphragm and the muscular coat of the jejunum of the rat. The reasons for the choice of these tissues are: (a) they possess different respiratory characteristics thus making it possible to illustrate the versatility of the diver technique; (b) other factors which might affect the results are eliminated or their effects reduced. The effect of

çell damage in the slice technique has recently been discussed by Field (12) and it is obvious that such effects might become seriously large with the minute slices used in the Diver. The tissues selected are of convenient thickness and only the cells at the edges are damaged by cutting.

The rat was killed by a blow on the head and the diaphragm placed in calcium-free Ringer-Locke solution at room temperature. After removing excess moisture by filter paper, about 0.3 mg of tissue were excised from the muscular region. The tissue sample was transferred by means of a glass needle to a small tared stoppered tube containing a drop of the same solution. To avoid loss of the solution on the tip of the needle, the tissue was first placed on the dry wall of the tube beside the medium drop. A sharp movement of the tube after stoppering causes the medium drop to cover the tissue.

The oxygen uptake and R.Q. of the diaphragm was determined by each of the three methods described above, the final arrangements of the diver contents being as shown in Fig. 1 b, c, and d. A modification of the technique of filling was necessary with pieces of tissue. The diver neck was filled with calcium-free Ringer-Locke solution down to the position to be finally occupied by the reaction ring. The tissue was transferred on the tip of a glass needle and floated down the column of solution until it rested on the lower meniscus. The excess solution was then pipetted off until the tissue remained suspended in a 1 μl. ring.

The O_2 uptake of the sample was determined for 30 minutes; the completed diver filling being as in Figure 1 b. The amount of tissue taken was such as would be expected to give a total O_2 uptake of about 0.1 μl. in the experimental period. The results obtained by Waterlow and Borrow (22) indicate that with homogenates and suspensions, provided the ring thickness employed did not exceed 1.1–1.2 mm, the diffusion of O_2 was not limiting with an uptake of 0.3 μl./hr. In the present study the thickness of ring plus tissue did not exceed this limit and the total O_2 uptake was less. An attempt was made to reduce the gas diffusion path by reducing the medium volume to 0.5 μl. This was abandoned for routine work since such a narrow seal containing tissue tended to rupture. The results obtained, however, fell within the same range as when 1 μl. of medium was used.

In these experiments the tissue was finally removed from the diver and the thickness of the slice estimated by measuring its area and assuming a specific gravity of one. The average sample thickness was 0.31 mm S. D. 0.065, and the average Q_{O_2} 4.0. S. D. 0.8. No significant correlation was found between the sample thickness and its Q_{O_2} in thirteen experiments. ($r = -0.503$, $P > 0.05$.)

TABLE V

Respiratory Quotient of Rat Diaphragm and Jejunum Muscular Coat.

Material	Method	QO_2 (μl/mg wet wt./hr.)	R.Q.	CO_2 Q_G (μl/mg wet wt./hr.)
Diaphragm.....	1st method	0.81	0.84	
		0.94	0.82	
		1.34	0.80	
		1.45	0.79	
		1.21	0.85	
		1.20	0.85	
	2nd method	0.91	0.84	
	P-K seal	0.92	0.85	
	correcting	1.55	0.80	
	for leak.	1.23	0.80	
	2nd method	0.99	0.85	
	MOBA seal	0.90	0.83	
		1.06	0.84	
		1.04	0.81	
		1.00	0.84	
		0.98	0.84	
		0.92	0.85	
		1.06	0.85	
Jejenum muscular coat	2nd method	0.81	0.85	0.97
	MOBA seal	0.92	0.87	0.99
		0.81	0.88	0.96
		1.34	0.90	1.02
		1.23	0.89	1.00
		1.18	0.88	1.05
		1.29	0.89	1.00
		1.86	1.03	0.94
		1.89	0.99	1.54
		1.23	1.04	0.87
		1.26	0.88	1.01

In the first method for the determination of R.Q. the procedure is as described for yeast. Complications arise in the second method, and therefore the complete procedure will be described. The divers were first flushed with the appropriate gas-mixture under water, and the KI and $KMnO_4$ reagents were placed as before. The neck of the diver was then filled with Ringer-bicarbonate medium to within one mm of the mouth, leaving a small gas

TABLE VI

Specimen Protocol for Experiment on Rat Jejunum Muscularis using second method. Moba Seal.

Control		
Weight tissue	0.311 mg	
Gas-Phase CO_2	0.416 µl	
Initial CO_2	0.405 µl	
Total CO_2 absorbed	1.150 µl	
Test		
Weight tissue	0.258 mg	0.229 mg
DV 30 min.	0.122 µl	0.099 µl
Final CO_2	0.297 µl	0.329 µl
Gas-Phase CO_2	0.484 µl	0.356 µl
Total CO_2 absorbed	1.327 µl	1.170 µl
CO_2 respired	0.136 µl	0.126 µl
CO_2 glycolysed	0.130 µl	0.116 µl
O_2 respired	0.158 µl	0.144 µl
QO_2 (µl/mg wet wt./hr.)	1.23	1.26
$\left.\begin{matrix} CO_2 \\ QG \end{matrix}\right\}$ (µl/mg wet wt./hr.)	1.04	1.01
R.Q.	0.87	0.88

space between the medium and the water in which the diver is submerged. The diver was then removed from the water, the tissue sample introduced and excess solution removed as above. Since it is necessary to have a constant amount of bicarbonate medium in the final reaction ring, the initial neck filling and the removal of excess solution must be quantitative. The errors in this procedure were shown to be not greater than those inherent in the apparatus. Bicarbonate free medium was employed in the weighing tube to avoid the addition of a variable amount of bicarbonate. The diver was then resubmerged and the neck above the reaction ring gently flushed with gas-mixture. The filling was completed and the determination carried out as described for yeast. Control correction values were calculated as described by Dickens and Šimer (10). The results obtained by the various methods are given in Table V and both Q_{O_2}'s and R.Q's are in good agreement with the figures given by Gemmill (13) obtained by the conventional Warburg method.

In the case of rat jejunum, selected for its high glycolytic activity, the muscular coat was obtained as described by Dickens and Weil-Malherbe (11). In order to minimise possible contamination with fat, the intestine was slit along its antimesenteric border, rinsed in buffer, the mucosa stripped

off, and samples taken from the cut edge of the muscular coat. The values obtained for oxygen uptake, R.Q., and glycolytic activity, in divers with MOBA seals, are given in Table V and a specimen protocol in Table VI. It will be seen that these fall within the range given by Dickens and Weil-Malherbe (11).

THE STUDY OF SINGLE ENZYME SYSTEMS USING TISSUE HOMOGENATES

Micro-homogenisers fitted with glass stoppers were constructed following the directions of Steele, Sfortunato, and Ottolenghi (21). In general, after homogenising about 1 mg. of liver in 10–50 μl. of the required concentration for three minutes at about 1 500 R.P.M's the tissue was reduced to a reasonably homogenous suspension. Microscopical examination immediately after homogenisation revealed nuclei and cytoplasmic inclusions which had been released during the process, and very few whole cells. All homogenates were prepared using ice-cold reagents, and the tube was immersed in an ice bath during homogenisation.

The Succinoxidase System

Succinoxidase was used as a test enzyme system since optimum conditions have been worked out by Schneider and Potter (19). Wide necked divers (1.0–1.3 mm^2) of volume about 10 μl. were used. The reagents were adjusted so that their molarity in the final reaction mixture was the same as that used by the above workers.

The procedure was as follows:

A liver lobe was excised and placed in ice-cold isotonic KCl prior to weighing. Excess aqueous solution was then removed by filter paper, and about 1 mg of tissue weighed into a stoppered homogenisation tube. After homogenisation in 50 μl. 8.5 per cent sucrose, one volume of homogenate was diluted with 2 volumes of medium containing 0.05 M Phosphate Buffer pH 7.4; 0.2×10^{-4} M. Cytochrome c; 0.2×10^{-3} M $CaCl_2$; and 0.2×10^{-3} M $AlCl_3$. Serial dilutions were made using $\frac{2}{3}$ strength medium. The divers were filled as in Fig. 1 e, succinate being omitted in controls. After allowing 20 minutes for equilibration, substrate and homogenate were mixed by downward displacement and the O_2 uptake followed at intervals for a period of 1 hour.

In Figure 3 the curves of O_2 uptake for various tissue dilutions are shown. Over the period of 1 hour the O_2 uptake was linear and the dilutions are in good stoichiometric agreement. The average value for succinoxidase activity of rat liver was 22.2 μl. O_2/hr/mg wet weight S.D. 2.9 which agrees well with the results of other workers.

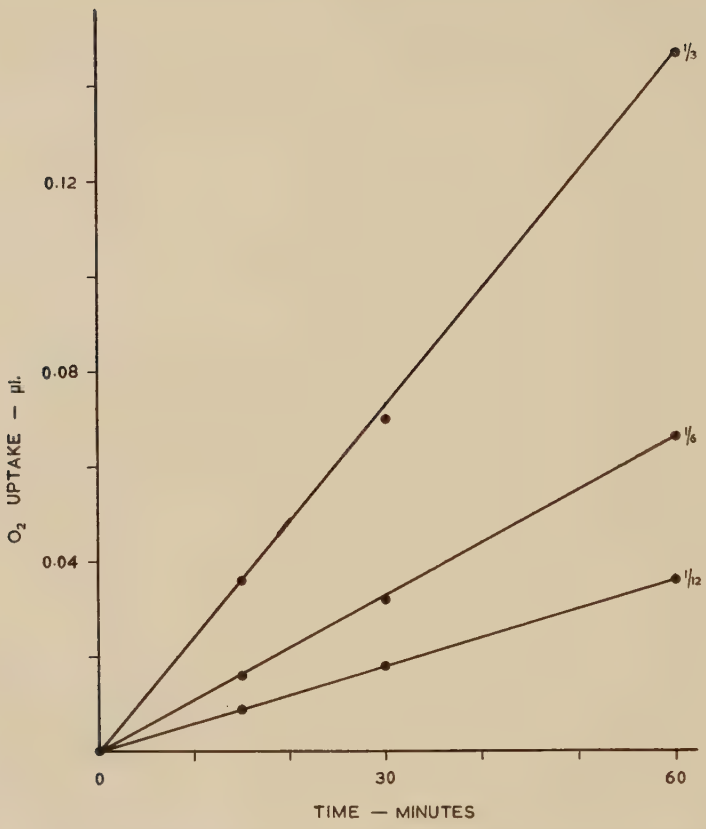


Fig. 3. Succinoxidase activity of rat liver homogenates. O_2 uptake for serial dilutions of homogenates.

When working with tissue samples of this magnitude, it is necessary to ascertain whether the small pieces of tissue taken for examination are representative of the whole liver. The succinoxidase activity was therefore determined using samples taken from two central positions and the periphery of the lobe, and also from central portions of two lobes from the same liver (mouse). Consistent results were obtained with samples taken from the central portions of the liver lobes (Q_{O_2} 50.0; S.D. 1.7) but the value given by the peripheral sample was low (Q_{O_2} 24.0).

The Transaminase System

The method of Ames and Elvehjem (1), employing the reaction:

aspartate + α ketoglutarate ⇄ glutamate + oxalacetate

was used. Excess aspartate and α ketoglutarate are added and the oxalacetate produced in a given time is estimated by the CO_2 evolved after the addition of aniline citrate.

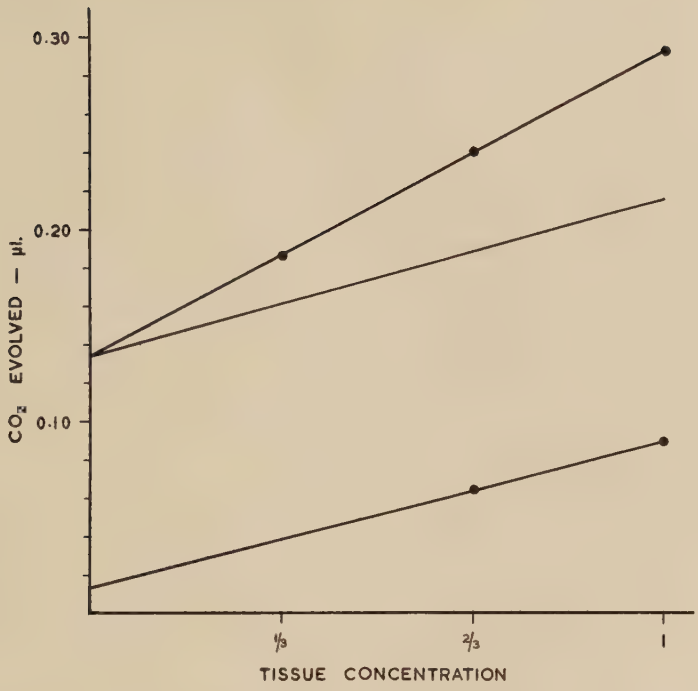


Fig. 4. Transaminase activity of rat liver homogenates. (Graphical representation of the Ames and Elvehjem method.)

The homogenate was prepared from about 1 mg of rat liver in 20 µl. isotonic sucrose. Long necked divers with volumes about 10 µl. were filled as shown in Fig. 1 f, the fortified homogenate consisting of 2 vols. sucrose homogenate; 1 vol. 0.5 M sodium potassium phosphate pH 7.3; 1 vol. 0.2 M sodium aspartate. In the control, α ketoglutarate was omitted. Determinations were made with three dilutions of homogenate; and two dilutions of homogenate were taken for controls. After 20 minutes' equilibration, homogenate and α ketoglutarate were mixed by upward displacement and the reaction allowed to proceed for 10 minutes. Aniline citrate was then mixed with the reaction ring by downward displacement. The CO_2 evolved was then determined, the maximum reading being attained in 10–20 minutes. The solubility correction factor in the aqueous solutions was taken as that for water. Typical dilution and correction curves are shown in Fig. 4. The average Q_T^{10} found using rat liver was 68.2 S.D. 5.0.

DISCUSSION

The present investigation has been concerned with the value of the Cartesian Diver for the study of the metabolism of mammalian tissue. The determination of oxygen uptake in the absence of CO_2 presents little difficulty, and the values of Q_{O_2} obtained for the tissues studied fall within the range published by other workers using the conventional Warburg apparatus.

The presence of CO_2 in the gas-phase of the diver leads to difficulties owing to leakage of the gas through the oil seals into the flotation medium; these have been ameliorated by replacing the usual paraffin-kerosene seal by one of mineral oil saturated with benzoic acid. The leakage rate was reduced to about $\frac{3}{4}$ per cent of the gas-phase CO_2 per hour, when the volume of oil used for the seal was 1 μl.

Linderstrøm-Lang and Holter (17) placed glass stoppers in the oil seal to reduce diffusion of CO_2 from the main gas volume (V–ω). The partial pressure of CO_2 in the *w* bubble will, in both cases, be reduced, resulting in a smaller loss through the medium seal and also reducing the initial unstable stage due to increasing amount of CO_2 being dissolved in the medium seal. Since the partial pressures of CO_2 on both sides of the oil seal are different some doubt would seem to exist as to the validity of the correction for CO_2 dissolved in the oil seal. The practical results obtained for CO_2 recovery, assuming that the oil is saturated with CO_2 at the partial pressure existing in the main gas-phase show, however, that no significant error is introduced when either the P-K or MOBA seal is used. Throughout the experiments the conditions for minimising errors suggested by Linderstrøm-Lang (15) have, as far as possible, been fulfilled, i.e. *w* has been kept small (about $0.1 \times V$;) and V_w of the order of $0.2 \times V$.

The experiments reported here are of short duration; for prolonged experiments the CO_2 leakage will lead to errors if the evolution of CO_2 is being measured, although partial correction should not be difficult. The range of substances dissolved in the oil in order to reduce CO_2 diffusion was not extensive. Similarly there are grounds for suspecting that reduction of the pH of the flotation medium might be a factor in reducing the loss of CO_2 from the diver; both these factors might repay further investigation.

Recently Claff and Tahmisian (8) have introduced an "hour glass" diver which employs two bulbs in order to facilitate mixing. In our experience the use of DC 200 obviates the need for specially constructed divers. In

addition to the increased volume, with its concomitant decrease in sensitivity, this type of diver is also not suitable for mixing by upward displacement.

Before the Diver technique can be generally applied to metabolic studies of slices of tissue, further work will be necessary. Experiments with the Warburg respirometer have shown that tissue slice thickness is a controlling factor in respiratory determinations (12). With the small pieces of tissue used in the diver, O_2 diffusion is unlikely to become limiting, but cell damage inherent in their preparation may introduce serious errors when such a tissue as liver is investigated. In the present investigation this source of error has been to some extent avoided by the use of tissue obtainable in sheets. When homogenates were used no difficulties were encountered providing the suspension was homogeneous.

Small tissue samples have been taken as being representative of the whole organ. This assumption seems to be justified since duplicate experiments from the muscular tissues employed gave essentially the same results. Further support is given by the succinoxidase activity determinations in which constant results were obtained from samples taken from the same lobe and also from different lobes of the same liver providing the periphery was avoided. It is probable that in some pathological conditions, a small sample would not be representative of the whole organ. Yet in conjunction with cytological examination, valuable information about metabolism in different localities of the same organ might be obtained. The value of the small sample is illustrated by the work of Anfinsen (2) who estimated the diphosphopyridine nucleotide content of the various layers of bovine retina.

SUMMARY

(1) The adaptation of the Cartesian Diver for use with mammalian tissues has been investigated.
(2) The behaviour of the Diver at 37° C. has been examined and found satisfactory.
(3) A mineral oil seal saturated with benzoic acid, substituted for the paraffin oil seal, has been found to reduce the loss of CO_2 from the Diver.
(4) Two methods for the determination of R.Q. of tissues have been adapted to the Diver, using yeast as a test material.
(5) The O_2 uptake, R.Q. and glycolytic activity of rat diaphragm, and the muscular coat of rat jejunum have been determined in the Diver.
(6) The methods of estimating succinoxidase and transaminase activity in homogenates prepared from 1 mg of tissue are described.

ACKNOWLEDGEMENTS

We wish to express our thanks to Professor B. S. Platt at whose suggestion this work was undertaken; to Dr. J. C. Waterlow for valuable criticism and advice; and to Professor Keilin for a gift of cytochrome c. Various samples of mineral oil were kindly supplied by the Shell Oil Company.

REFERENCES

1. Ames, S. R., and Elvehjem, C. A., *J. Biol. Chem.*, **166**, 81 (1946).
2. Anfinsen, C. B., *J. Biol. Chem.*, **152**, 279 (1944).
3. Boell, E. J., Koch, H., and Needham, J., *Proc. Roy. Soc., London, Ser. B*, **127**, 374 (1939).
4. Boell, E. J., and Needham, J., *Proc. Roy. Soc., London, Ser. B*, **127**, 356 (1939).
5. —— *Proc. Roy. Soc., London, Ser. B*, **127**, 363 (1939).
6. Boell, E. J., Needham, J., and Rogers, V., *Proc. Roy. Soc., London, Ser. B*, **127**, 322 (1939).
7. Boell, E. J., and Nicholas, J. S., *J. Exptl. Zool.*, **109**, 267 (1948).
8. Claff, C. L., and Tahmisian, T. N., *J. Biol. Chem.*, **179**, 577 (1949).
9. Dickens, F., and Šimer, F., *Biochem. J.*, **24**, 905 (1930).
10. —— *Biochem. J.*, **25**, 973 (1931).
11. Dickens, F., and Weil-Malherbe, H., *Biochem. J.*, **35**, 7 (1941).
12. Field, J., Methods in Medical Research, ed. V. R. Potter, Vol. 1., Chicago, 1948.
13. Gemmill, C. L., *Bull. Johns Hopkins Hosp.*, **68**, 329 (1941).
14. Holter, H., *Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim.*, **24**, 399 (1943).
15. Linderstrøm-Lang, K., *Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim.*, **24**, 333 (1943).
16. Linderstrøm-Lang, K., and Glick, D., *Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim.*, **22**, 300 (1938).
17. Linderstrøm-Lang, K., and Holter, H., *Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim.*, **24**, 105 (1942).
18. Needham, J., Rogers, V., and Shen, S., *Proc. Roy. Soc., London, Ser. B*, **127**, 576 (1939).
19. Schneider, W. C., and Potter, V. R., *J. Biol. Chem.*, **149**, 217 (1943).
20. Schwartz, S., *Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim.*, **27**, 79 (1949).
21. Steele, R., Sfortunato, T., and Ottolenghi, L., *J. Biol. Chem.*, **177**, 231 (1949).
22. Waterlow, J. C., and Borrow, A., *Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim.*, **27**, 93 (1949).

SOME PHYSICO-CHEMICAL PROPERTIES OF SPONTANEOUS MOUSE ENCEPHALOMYELITIS VIRUS, STRAIN FA

H. LEYON

Institute of Biochemistry, University of Uppsala, Sweden

Received August 26, 1950

Spontaneous mouse encephalomyelitis virus was discovered by M. Theiler in mice (13, 14) and has been subsequently called Theiler's virus (Theiler's original strain, TO). Later, strain FA of higher virulence than TO was isolated in Theiler's laboratory (15).

The physico-chemical properties of Theiler's virus, strain FA, have been studied by Theiler and Gard (15). In ultrafiltration experiments the virus was found to pass collodion membranes of all grades down to 35 mμ average pore diameter but failed to pass through a membrane of 27 mμ average pore diameter.

The sedimentation constant obtained by the separation cell method was reported by Gard (4) to be 152 S. By the optical method somewhat different values were found by the same author for solutions of different concentrations of salt, the sedimentation constant varying between 150 S and 191 S. These figures refer to preparations from mouse brains. For the same material a diffusion constant, D_{20}, of $0.32 \cdot 10^{-7}$ cm^2/sec was reported. Electron micrographs of Theiler's virus, strain FA, have been published by Tiselius and Gard (16) and by Gard (5, 6). They all show filamentous structures similar to the ones published in the same reports on intestinal material. Later, only spherical particles about 28 mμ in diameter were found in electron micrographs of preparations of mouse brains (7).

The sedimentation constant found by Gard was ascribed to the filaments found by him in the electron micrographs (16, 5, 6). It therefore seemed of interest to know the sedimentation constant of the virus particles occurring as spheres of about 28 mμ diameter in the micrographs obtained later.

The present communication summarizes the results of studies of (a) the sedimentation in the ultracentrifuge of purified preparations of spontaneous mouse encephalomyelitis virus, strain FA, (b) the absorption in ultraviolet light of the same preparations and (c) the pH-stability of the virus.

It would have been desirable to know the diffusion constant of our purified preparations. It has, however, so far not been possible to collect enough material for such a measurement.

MATERIAL

The virus used in the experiments was originally obtained from Theiler's laboratory. It had undergone a number of passages in the course of Gard's work (4). A few brains stored in 50 per cent glycerol at +4° C since March 1943 were prepared and passed through mice in June 1947. The inoculated mice developed the symptoms described as typical for FA (15, p. 50). Since then series of experiments of the pH-stability of the virus and on adsorption methods have been carried on until the end of 1949. The brains left from one experiment and not used immediately in the following ones were stored at —15° C. The residues from purified preparations were stored under ether in the same way. In the autumn of 1949 all stored material was purified in batches of 150–300 brains. The partly purified preparations were taken together in one final purification. The physico-chemical measurements described were made on this last preparation. The different batches pooled together in the last preparation contained virus which had undergone a different number of passages. This number varied from 20 to about 60. No changes in virulence nor symptoms caused were observed between the different preparations.

METHODS OF PURIFICATION

An especial study of methods of purification was not included in these researches. It is perhaps sufficient to point out that very little appears to be known concerning the events taking place during the common procedures used for isolation of the virus.

The methods used for concentrating the virus have been reported elsewhere (7). The preparations called "crude" only were centrifuged at a low speed and treated with $\frac{1}{5}$ volume of ether over night after their pH had been adjusted to 7–8 by means of a sodium phosphate solution. To get a more concentrated and partly purified preparation it was found most convenient to precipitate at a low pH and resuspend in a small volume, as was done by Bourdillon (1) on preparations of SK poliomyelitis virus. The pH was lowered to 4.5 in the first experiments but after the flocculation of the virus at different pH-values had been studied, the solutions were acidified to pH 4.1–4.3. Further purification of the virus was achieved by freezing the concentrated suspensions for some time, usually a couple of weeks, followed by centrifugations at low and high speed as reported in (7). The yield at this point of the purification procedure was usually $\frac{1}{5}$–$\frac{3}{4}$ of the original amount, calculated as number of infectious doses, ID_{50}.

The most concentrated suspension which was used for determining the sedimentation constant and the light absorption curve had been purified by treatment with ether, precipitation at a low pH, centrifugation at low and high speed, precipitation in $\frac{1}{3}$ saturated ammonium sulphate solution, and freezing of the resuspended virus

at —15° C, followed by centrifugation at low and high speed. The last mentioned procedures—freezing and sedimentations—were repeated twice.

The centrifugations were at first done at 16,000 revolutions per minute up and down followed by 27,000 r.p.m. during 1 ½ hours. Later on the preparations were centrifuged at 10,000 r.p.m. up and down and 20,000 r.p.m. for 1 ½ hours. The distance from the bottoms of the tubes to the centre of rotation was 10 cm. The highly purified final suspension gave pellets as a rather loose jelly after the centrifugation at a high speed. In less pure preparations the pellets are densely packed.

All purification work was done at 0–4° C. When stored even if only for one night at −4° C the preparations always contained ether to prevent bacterial infection.

MEASUREMENTS OF VIRUS ACTIVITY

Serial tenfold dilutions were made in 0.9 per cent sodium chloride solution. One pipette was used for each dilution. The instruments and glasses and the solutions for dilution were sterile. About 0.025 ml of each dilution were injected intracerebrally into 5 white mice. Mice showing spasms or pareses were taken as infected. The 50 per cent end point of infection was calculated according to Reed and Muench (11). The titers are given as log number of infectious doses per milliliter of the undiluted preparation, the infectious dose, ID_{50}, being the amount of virus which gives infection in 50 per cent of the inoculated mice.

MEASUREMENTS OF SEDIMENTATION IN THE ULTRACENTRIFUGE AND ULTRAVIOLET LIGHT ABSORPTION

From the stored material a final preparation was obtained as described above. It was concentrated with the preparative ultracentrifuge to about 0.2 ml. With the kind help of Dr. K. O. Pedersen a new microcell for the "equilibrium centrifuge" was made and tested. Its volume was 0.11 ml. In this cell the preparation was run at 12,000 r.p.m. at 20° C. The distance, x_0, from the centre of rotation to the meniscus was 7.25 cm. The Lamm scale method was used to follow the sedimentation. The solution was 0.01 M with respect to sodium phosphate and its pH 7.9. An easily observed boundary was obtained which reached the bottom of the cell in about 90 minutes. The sedimentation diagrams show only one distinct component, the sedimentation constant of which was determined to be 151 S.

The virus was resuspended and the solution diluted with an equal volume of phosphate buffer of the same kind. The preparation was then again spun in the ultracentrifuge. In this way the sedimentation constants were obtained for the two-, four- and eightfold dilutions of the original preparation. The values 154, 159 and 159 S were obtained. The interpretation of the data

from the run giving 154 S is somewhat uncertain. It is possible that value 154 S might be lower.

If dn/dc (n = refractive index, c = concentration) for the substances in the solution was assumed to be the one observed for many proteins (i.e. $\Delta n = 19 \cdot 10^{-6}$ at 546 mμ for 1 per cent solution) the concentration of protein in the undiluted solution was determined to 1.2 per cent with a probable error of about ± 0.3 from measurements of the refractive index. From the sedimentation diagrams the concentration of the sedimenting substance was determined to be about 0.5 per cent, based on the same assumption. Obviously the preparations contained other material than the sedimenting particles.

A small part of the original solution was taken and diluted with distilled water 65/1. The pH of the diluted solution was 7.9. Its absorption in ultraviolet light was measured with a Beckman spectrophotometer, model DU. The absorption curve was the one typical for nucleoproteins (See Fig. 2). Also the curve obtained when diluting the original solution with 0.1 M NaOH is similar to that given e.g. by nucleoproteins from calf thymus (3).

The preparation was also investigated in the electron microscope. Spherical particles of the same kind as observed earlier (7) dominated the picture. When the preparation was diluted there appeared also to be a bottom layer of "lower molecular" material.

By biological titrations log ID_{50}/ml original solution was determined to be 9.5.

The different samples of the highly purified preparation were pooled and the mixture was diluted to about 7 ml with 0.01 M phosphate buffer solution of pH 7.5 and spun at low and high speed in the Beam's centrifuge. The procedure was repeated once. No pellets were seen after the last centrifugation. The solution was carefully drawn off except for about 0.2 ml. The preparation was left in the cold room for a few hours and was then studied anew in the ultracentrifuge, the Beckman spectrophotometer and in the electron microscope.

A study of the bottom layer of the micrographs showed that this preparation contained less contaminants than the first one. The ultracentrifugation gave the sedimentation constant 163 S (diagram Fig. 1). The concentration of sedimenting substance was calculated from the sedimentation diagrams to be about $\frac{1}{3}$ of the concentration of the original preparation before the last purification. The activity was determined to be log ID_{50}/ml = 9.0, i.e. a decrease of 0.5 in log activity, which is in agreement with the calculated concentration. Since only about 0.01 ml of the first preparation had been

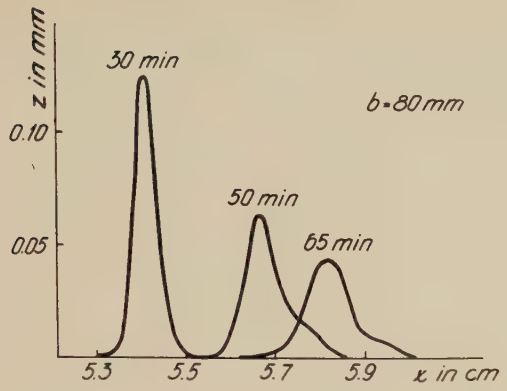


Fig. 1. Sedimentation diagrams for a purified preparation of spontaneous mouse encephalomyelitis virus (z = displacement of scale lines, x = distance from the centre of rotation b = scale distance. For each exposure is given the time elapsed after the rotor reached full speed.)

used for biological titration a great part was obviously lost during the last centrifugations. The virus particles seem to be rather unstable in the purified preparations. The extinction curve in ultraviolet light (Fig. 2) was of the same kind as that of the first preparation. Referring the extinction to the undiluted preparations of 0.2 ml, it was found that the extinction was 5 times higher before the last purification than afterwards. Since the extinction at 2,600 Å decreased significantly more than the concentration of the sedimenting substance during the purification, also the contaminants in the first preparation had a remarkable absorption at 2,600 Å. These contaminants might have consisted of virus, which was destroyed to a high degree.

The last purified preparation was diluted with an equal volume of phosphate buffer as before and was spun in the ultracentrifuge, giving the sedimentation constant 161 S.

Another preparation of virus purified by a procedure including treatment with ficin, was spun in the ultracentrifuge. The preparation contained about 0.2 per cent sedimenting substance and its pH was 7.5. The sedimentation constant was determined to be 159 S. When diluted with an equal volume of 0.01 M phosphate buffer solution of pH 7.5, the solution was spun again in the ultracentrifuge giving the sedimentation constant 164 S.

Thus the sedimentation constant was determined to be 160–165 S, the value 151 S for the highest concentration being significantly lower than those of more diluted preparations (Fig. 3).

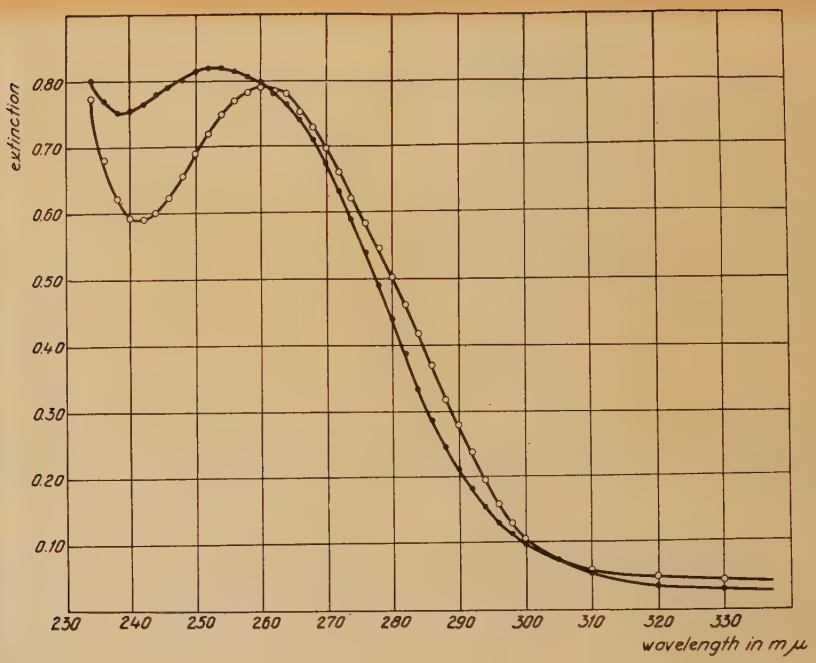


Fig. 2. Extinction curve for a purified preparation of spontaneous mouse encephalomyelitis virus, strain FA. (Open circles pH 7.9, filled circles 0.1 M NaOH.)

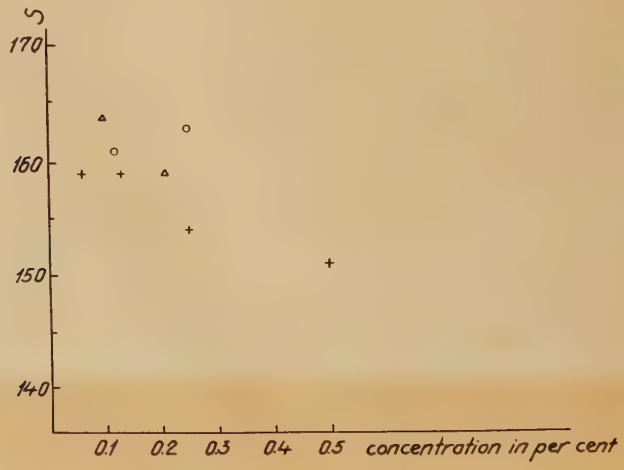


Fig. 3. Sedimentation constants for different preparations of spontaneous mouse encephalomyelitis virus, strain FA (+ a series of dilutions of a highly purified suspension, of the same preparation purified by two more centrifugations, Δ a preparation purified by a procedure including treatment with ficin).

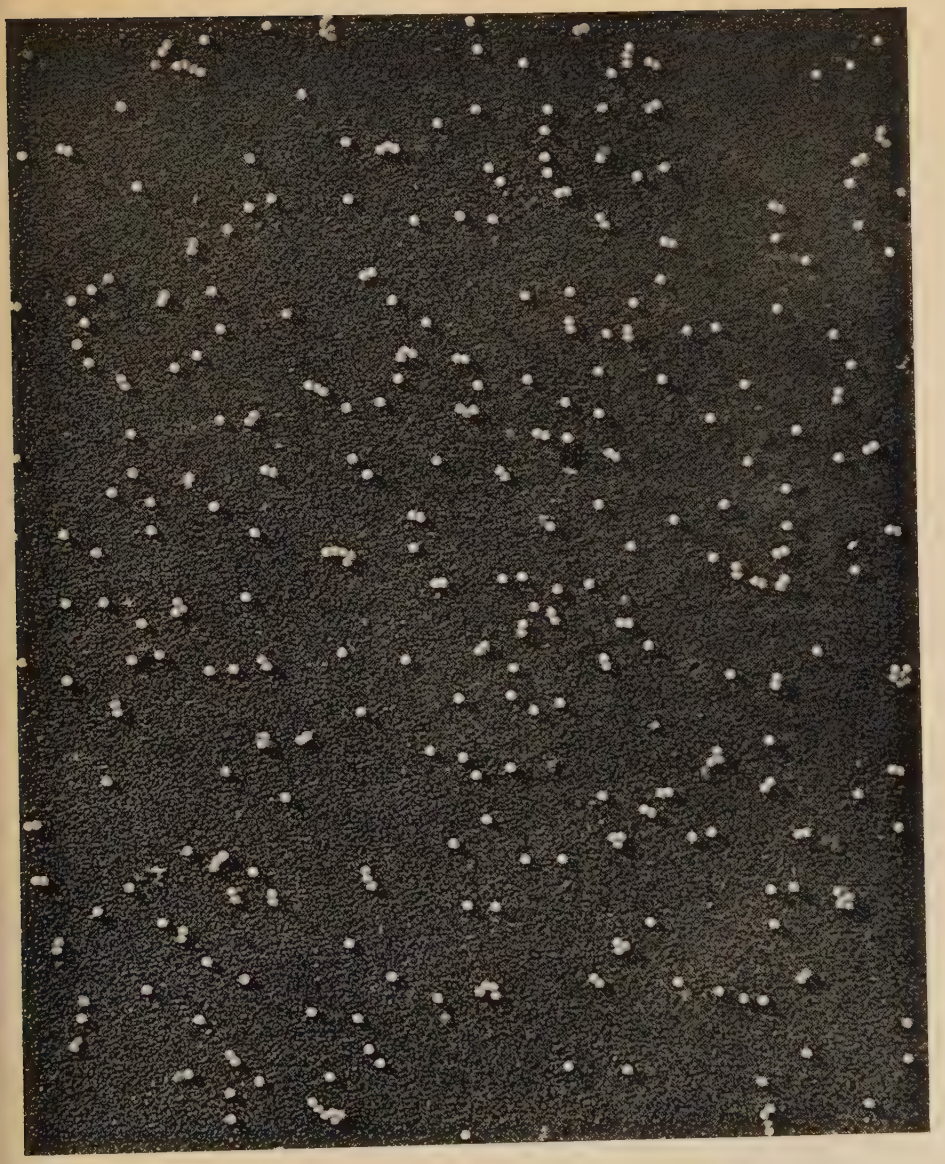


PLATE I. Electron micrograph of a highly purified preparation of spontaneous mouse encephalomyelitis virus, strain FA, on a collodion membrane covered with beryllium, the preparation shadowed with gold-manganin. 42,500 ×.

PLATE II. Electron micrograph of the same preparation as in pl. I diluted 5/1. 42,500 ×.

AGGREGATION OF THE VIRUS PARTICLES IN SOLUTION

The sedimentation diagrams, e.g. the one in Fig. 1, indicate that the solution also contained faster sedimenting material than the main component. Since only spheres of the kind described could be found in the micrographs there is a possibility that they were to some extent aggregated in solution. Ribi and Rånby (12) attempted a study on the aggregation of particles in solution by electron microscopy. By covering the supporting membrane with a thin layer of beryllium they got the particles in solution adsorbed on the membrane when the rest of the solution was sucked off. Aggregates were adsorbed as such. The ratio of number of aggregated particles to number of not aggregated particles was about the same in any one dilution in a graded dilution series.

On this basis serial 5-fold dilutions were made of the best purified preparation of Theiler's virus. Collodion membranes were covered with a layer of beryllium, as thin as possible, in the shadow casting apparatus. Drops of the virus solutions were found to wet such membranes, which is not the case with collodion surfaces. When the solution was sucked off a very thin layer was left which dried quickly. The preparations were shadowed with gold-manganin in the usual manner. The electron micrographs (Pl. I and II), interpreted according to the theory described, may indicate aggregates, each consisting of a few virus particles, which support the view that the fast sedimenting component in the sedimentation diagrams may be ascribed to such aggregates.

pH-STABILITY-STUDIES

Theiler and Gard reported that spontaneous mouse encephalomyelitis virus, strain FA, has one optimum of stability at pH 8 and another at about 3.3 (15). Similar inactivation curves i.e. with more than one zone of stability have been reported also for foot-and-mouth disease by Pyl (10) and for equine encephalitis virus, Eastern strain, by Finkelstein, Marx, Beard and Beard (2). The results were obtained for foot-and-mouth disease on crude lymph from infectious vesicles, for equine encephalitis virus on extracts of whole diseased chicken embryos and for spontaneous mouse encephalomyelitis virus on crude brain tissue extracts.

No definite explanations could be given for the special type of pH-stability of these three viruses, but different hypotheses were suggested. Theiler and Gard pointed out that there might be some connection between the floccula-

tion of the virus and the variation in stability with pH, Finkelstein *et al.* suggested that the factor causing inactivation at intermediate pH-values might be an enzyme with optimal effect at that pH. When the work here reported was started this last hypothesis seemed of special interest. From that point of view it was considered important to compare the inactivation of crude and purified virus preparations of varying acidity. The flocculation of the virus in acid solutions was studied and compared with the inactivation. The influence of the temperature on the inactivation at pH 5.1 was also studied.

Since the method of biological titration is not very accurate and the number of mice available was limited, the results were not always as accurate and could not always be as well verified as would have been desirable. Thus experiments on the influence of the temperature on the inactivation at pH 5.1 of partially purified preparations were started on the conception that there was a maximum of inactivation of such extracts established for that pH. Later experiments, however, gave results contradictory to the first ones. Even if prepared in the same way crude virus suspensions varied rather much in pH-stability for reasons which could not be found in these experiments. Considering the limitations of the methods, all our experiments taken together do not show any certain maximum in inactivation of the virus at pH about 5. In the main the inactivation on the acid side is faster the lower the pH. There is often, however, at pH about 4 some slowing down of the inactivation. This effect may cause a smaller inactivation at pH 4 than at pH 5 but usually the decrease in titer is about the same at these pH-values or somewhat larger for pH 4. The retarding effect on the inactivation at pH about 4 is probably due to the flocculation at what might be the isoelectric point of the virus.

In spite of the lack of continuity between some of the experiments and the incompleteness of the study of the pH-stability, the results reported below may be of some interest.

The same sort of material was used in the experiments on pH-stability as in the sedimentation measurements. Since experiments of this kind were going on from July 1947 to December 1949 the virus in the different samples had undergone a different number of passages. The rate of inactivation was studied in the following way: In each of a series of glass bottles were mixed one volume of virus suspension and 9 volumes of buffer solution of different pH but the same ionic strength, $\mu = 0.1$. The undiluted virus suspension was titrated in mice at the beginning of the experiment. The bottles were rotated in a water thermostat. Measurements of pH were made with a glass electrode before and after the inactivation period. In the range of pH 9–12 the measurements were checked with a hydrogen electrode. At the end

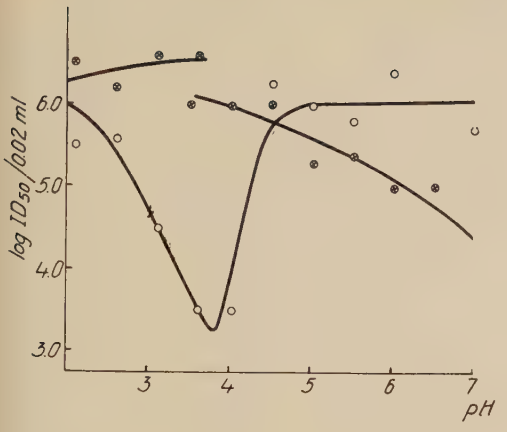


Fig. 4.

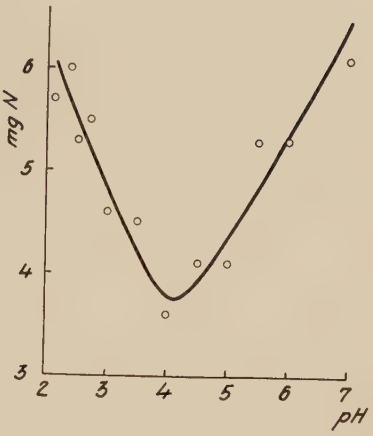


Fig. 5.

Fig. 4. The flocculation of spontaneous mouse encephalomyelitis virus, strain FA, against pH. (Open circles the titer values of the supernates, crossed circles those of the sediments.)
Fig. 5. The flocculation at different pH of the nitrogen-containing material in a 10 per cent suspension of calf brain in 0.3 per cent sodium chloride solution after treatment with ether. Ordinate: mg N in 100 ml supernate.

of the period of inactivation the solutions were neutralized to pH 7.0–7.5 with 0.1 M NaOH and were then kept at 0° C until they were titrated in mice. The amount of inactivation is given as the difference between the titer of a neutral control suspension of pH 7.5 and the titer of the sample after inactivation. In most of the experiments acetate-phosphate-glycine buffer solutions according to Northrop and Kruif (8) were used. Later the inactivation was studied in veronal and tartrate buffer solutions on the acid side and in glycine buffer on the alkaline side. In separate experiments it was found that the inactivation rate at pH about 5 was the same in these buffers. The same end point titers were obtained for neutralized and non-neutralized suspensions. There was however a shortening of the incubation time when acid solutions were injected.

In crude preparations there is an easily observed flocculation at pH 3–6. From purification work it was known that the virus is precipitated at pH 4–4.5 (4, 1). To study the flocculation of the virus further the following experiment was made. Crude virus suspensions were mixed with 9 volumes of Northrop-Kruif buffer solutions of different pH and the mixtures were spun 10,000 r.p.m. up and down in the Beam's centrifuge immediately after mixing. The supernates were separated from the sediments which were resuspended in distilled water to the same volumes as the supernates. All the solutions were then neutralized and titrated in mice. Two similar

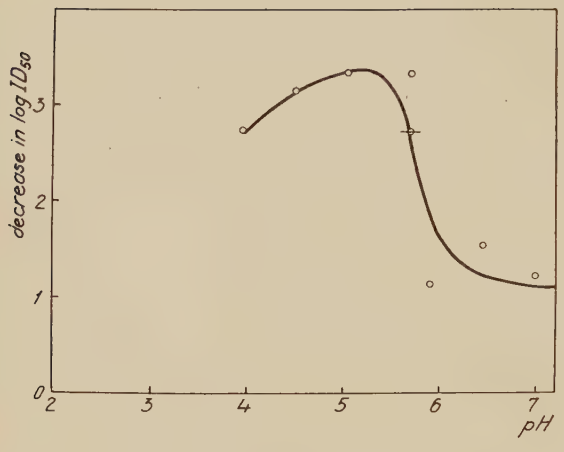


Fig. 6.

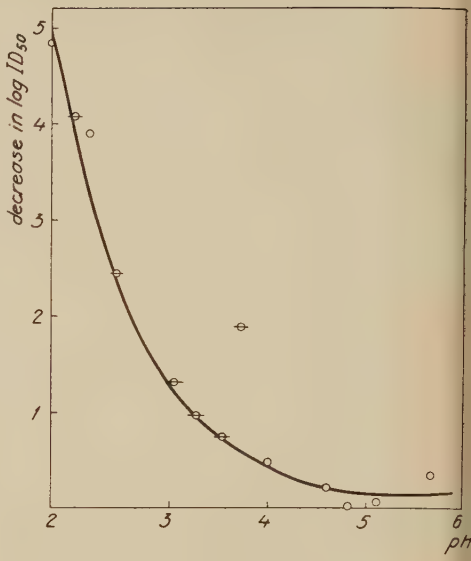


Fig. 7.

Fig. 6. The inactivation of a preparation of spontaneous mouse encephalomyelitis virus, strain FA, partially purified by ether, precipitation at a low pH, salting out and sedimentation in the ultracentrifuge. Temperature 33° C, time 36 hours. Northrop-Kruif buffer solutions were used.

Fig. 7. The inactivation of a crude suspension of spontaneous mouse encephalomyelitis virus, strain FA, in Northrop-Kruif buffer solutions. Temperature 26° C, time 29 hours.

experiments were made, one for pH 0–4, another for pH 3.5–7.0. The results are reported in Fig. 4, where log ID_{50} per inoculum is plotted for sediment and supernate against pH.

For a comparison brain tissue from a healthy calf was treated in the same way as the infected mouse brains. After centrifugation of samples of varying pH, nitrogen determinations were made on the supernates. As shown in Fig. 5 the proteins in this brain tissue extract were flocculated in the same way as was the virus in preparations from infected mouse brains.

In the figures 6–9 the results are reported of some typical inactivation experiments. The horizontal lines through the points give the change in pH during the experiments. Both crude and highly purified preparations are unstable above pH 10 (Fig. 9). Crude and partly purified preparations sometimes gave inactivation curves similar to Fig. 6. Most of the experiments, however, gave results as shown in figures 7 and 8 as reported above.

In fig. 10 the influence of the temperature on the rate of inactivation at pH 5.1 is shown.

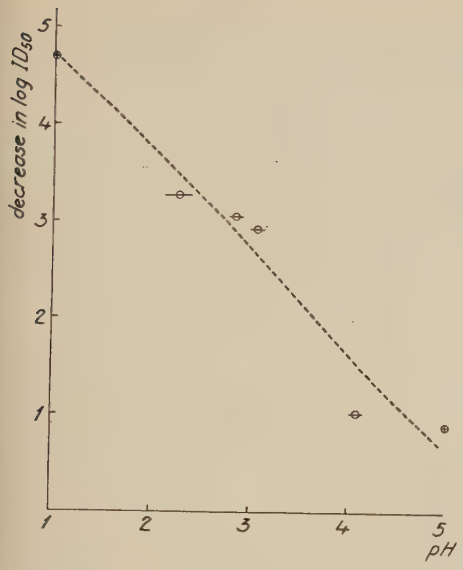


Fig. 8.

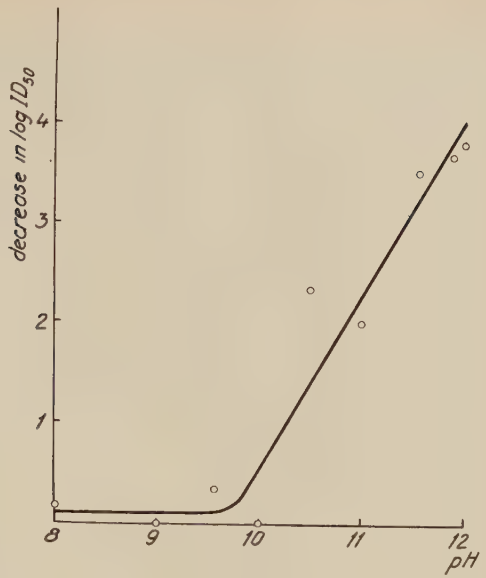


Fig. 9.

Fig. 8. The inactivation in tartrate (pH 1–3) and Northrop-Kruif (pH 4–5) buffer solutions of a preparation of spontaneous mouse encephalomyelitis virus, highly purified by ether treatment, precipitation at a low pH, freezing and repeated centrifugations. Temperature 33° C, time 21 hours.

Fig. 9. The inactivation in a glycine buffer solution of ionic strength 0.1 of a preparation of spontaneous mouse encephalomyelitis virus purified as described under Fig. 8. Temperature 31° C, time 12 hours.

The virus preparations used in these experiments were all partly purified by treatment with ether, precipitation at a low pH and centrifugation at low and high speed. One experiment was made for each temperature. 9 volumes of phosphate buffer solutions of pH 5.0 and ionic strength 0.1 were mixed with one volume of virus suspension. The pH of the mixture was about 5.1 but changed somewhat towards 5.2 during the experiments. The mixture was divided in 5 samples which were placed immediately into the thermostat where they were rotated in order to keep the contents well mixed. As soon as they attained the temperature of the bath one of the bottles was cooled under running tap water to about 10° C. The solution was neutralized and kept at 0° C until it could be titrated in mice. After different times had elapsed the other samples were treated in the same way.

It can be seen that the decrease in log concentration of the virus was direct proportional to time, the inactivation thus following the formula for a monomolecular reaction. There was no optimum of temperature for the inactivation. From Fig. 9 F the activation energy for the inactivation of the virus at pH 5.1 in phosphate buffer solution of ionic strength 0.1 was calculated as 34,500 calories per "Mol."

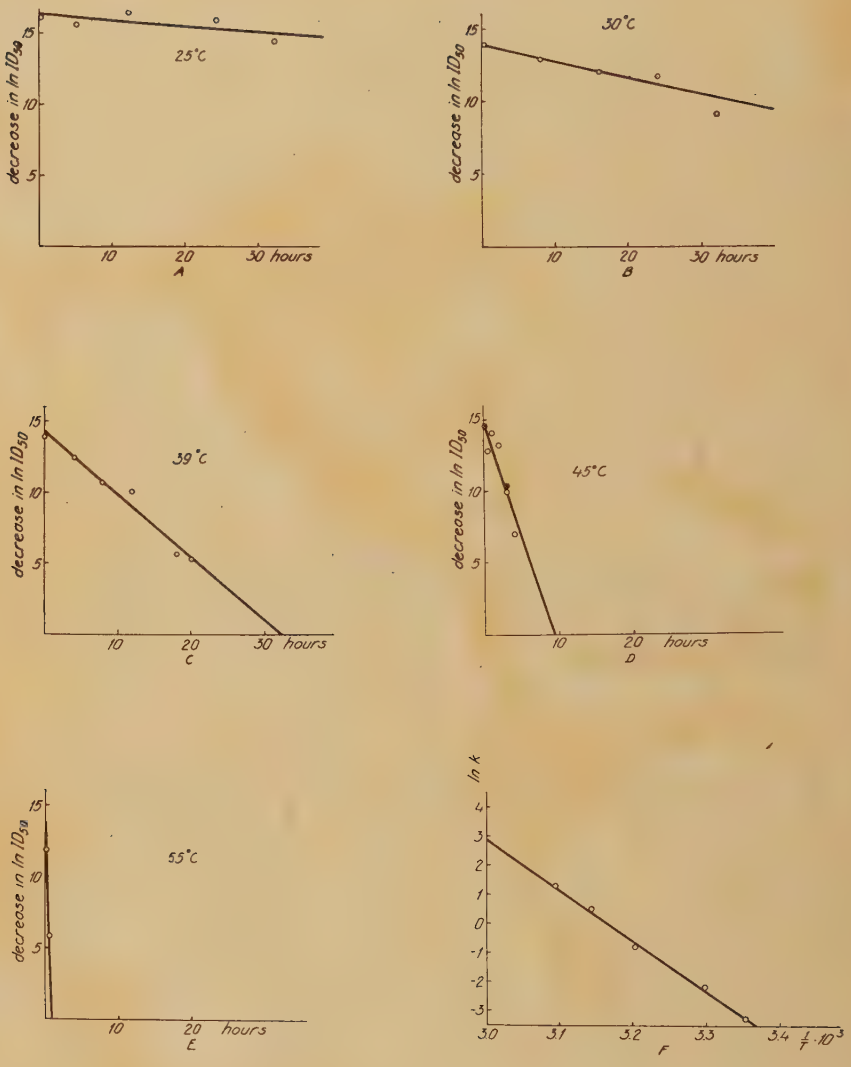


Fig. 10. The inactivation in phosphate buffer solutions of ionic strength 0.1 and pH 5.1–5.2 of preparations of spontaneous mouse encephalomyelitis, strain FA, partly purified by treatment with ether, precipitation at a low pH and centrifugations. In F the logarithms of the constants, k, of proportionality from A–E are plotted against the inverted values of the absolute temperature, T.

Plate III. Electron micrograph of a purified preparation of spontaneous mouse encephalomyelitis virus, strain FA, on a collodion membarane. 34,000 ×.

DISCUSSION

The values found for the sedimentation constant agree fairly well with the ones reported by Gard (4). Of special interest is the good agreement with the value 152 S obtained by him by the separation cell method for the carrier of the virus activity.

In the electron microscope all the preparations here described showed spherical particles of the kind reported earlier (7). Theoretically unhydrated spheres of 30 mμ diameter with a sedimentation constant of 160 S should have a density of 1.3. This value is in good agreement with values reported for viruses and proteins. Thus the sedimentation constant found, with the facts so far known, should be ascribed to the spherical particles in the electron micrographs. It may be mentioned for a comparison that bushy stunt virus is reported to give round images of 26 mμ diameter in the electron micrographs and to have a sedimentation constant of 132 S (9).

As for the filtration end point of 27 mμ average pore diameter (15) it seems very probable that the ratio particle diameter to limiting pore diameter is about 1 for Theiler's virus.

Theoretically the spherical particles of 28 mμ diameter should have a diffusion constant of $1.5 \cdot 10^{-7}$ cm²/sec. The value reported by Gard, $0.32 \cdot \cdot 10^{-7}$ cm²/sec., cannot reasonably be ascribed to these particles. Regarding the description of Gard's experiment (4, p. 133) it does not seem possible to attribute any value to the result of that measurement. Since the earlier electron micrographs showing filamentous particles in material from brains could not be verified even with a purifying technic similar to the one then used, there seems to be little reason so far to consider Theiler's virus as occurring in other form than the one having the spherical particles here described. The calculations presented here are of course hypothetical, for example hydration was not taken into consideration. They show however that there is no discrepancy so far between the results of the measurements from sedimentation and later electron microscopy.

SUMMARY

The sedimentation constant of spontaneous mouse encephalomyelitis, strain FA, was determined to be 160–165 S.

The sedimentation constant found is ascribed to the particles giving round images of 28 mμ diameter in the electron micrographs. The virus particles in the solutions are considered to be aggregated in clumps to some degree.

The purified virus preparations gave an absorption curve in ultraviolet light similar to the one given by nucleoproteins.

The pH-stability in crude and purified suspensions is described.

The thermal inactivation of the virus at pH 5.1 followed the formula for a monomolecular reaction and the activation energy of this reaction was determined as 34,500 calories per "Mol".

ACKNOWLEDGEMENTS

The work on Theiler's virus was financed by grants from the Swedish Natural Science Research Council and the Swedish Medical Research Council. It was supervised by Professor A. Tiselius at whose institute most of the work was done. The investigation on the pH-stability was initiated by Professor S. Gard who also for some time provided working facilities. Part of the work was done in the institutes of Professors T. Svedberg, S. Claesson, J. Reenstierna and S. Löfström. Miss G. Jandal did preparative work, Dr. E. Ribi and Mr. G. Eklund gave information and advice about the electron microscopical technic, and Mr. E. Hellman and Miss A. L. Norling about the theory and application of the ultracentrifugation technic. To all these persons this writer wishes to extend his sincere thanks.

REFERENCES

1. Bourdillon, J., *Arch. Biochem.*, **3**, 285 (1944).
2. Finkelstein, H., Marx, W., Beard, D., and Beard, J. W., *J. Infectious Diseases*, **66**, 117 (1940).
3. Frick, G., *Biochim. et Biophys. Acta*, **3**, 103 (1949).
4. Gard, S., *Acta Med. Scand., Suppl.*, **143** (1943).
5. —— *Klin. Wochschr.*, **16/17**, 315 (1943).
6. —— *Arch. ges. Virusforsch.*, **3**, 1 (1943).
7. Leyon, H., and Gard, S., *Biochim. et Biophys. Acta*, **4**, 385 (1950).
8. Northrop, J., and de Kruif, P. H., *J. Gen. Physiol.*, **4**, 639 (1922).
9. Price, W. C., Williams, R. B., and Wyckoff, R. W. G., *Arch. Biochem.*, **9**, 175 (1946).
10. Pyl, S., *Z. physiol. Chem.*, **244**, 209 (1936).
11. Reed, J. L., and Muench, H., *Am. J. Hyg.*, **27**, 493 (1938).
12. Ribi, E., and Rånby, B., *Experientia*, **6**, 27 (1950).
13. Theiler, M., *Science*, **80**, 122 (1934).
14. —— *J. Exptl. Med.*, **65**, 705 (1937).
15. Theiler, M., and Gard, S., *J. Exptl. Med.*, **72**, 50 (1940).
16. Tiselius, A., and Gard, S., *Naturwiss.*, **30**, 728 (1942).

STUDIES ON ISOLATED CELL COMPONENTS
III. A CYTOLOGICAL STUDY OF THE EFFECTS OF HEPARIN ON ISOLATED NUCLEI[1]

HENRY STOUTTE ROBERTS and NORMAN G. ANDERSON[2]

Department of Zoology, Duke University, Durham, N.C.

Received August 24, 1950

INTRODUCTION

The addition of heparin to isolated rat liver nuclei results in the release of desoxyribonucleic acid (DNA) or a DNA-protein complex as evidenced by viscosity changes and by chemical analysis (1, 2). These observations are of interest in view of the reported inhibition of cell division by heparin and heparin-like substances (3, 4) and the occurrence of hyperheparinemia in fatally irradiated animals (5). The following study was undertaken in an effort to gain further knowledge of the effect of heparin on the isolated nucleus through the application of stains to fresh preparations and by the use of the Feulgen reaction.

METHODS

Studies with unfixed nuclei. Nuclei were isolated by differential centrifugation from fresh rat liver in a sucrose-phosphate buffer at pH 7.1 (6). Since it has not been possible to eliminate the last traces of cytoplasmic contaminants from nuclear preparations, it was important to be able to identify and compare material of both nuclear and cytoplasmic origin in all preparations. Crude nuclear preparations were therefore used in order that cytoplasmic material would be regularly present and readily identified in both control and experimental preparations.

The fresh material was examined in the unstained condition or with methylene blue or methyl green, or with a modification of the Pappenheim pyronin-methyl green mixture. The latter was found useful for routine observations while making preparations.[3]

---

[1] Supported in part by a grant from the Duke University Research Council.

[2] Atomic Energy Fellow.

[3] A satisfactory mixture for vital staining consists of 0.17 g methyl green, 0.06 g pyronin, and 100 ml of 95 per cent ethyl alcohol. One drop is dried on each slide.

The effect of heparin and of basic dyes on isolated nuclei was studied by inducing solutions containing these substances to flow past the nuclei under the cover slip. All heparin preparations used had an anticoagulant activity of 100 Toronto units per milligram and were commercial dry beef lung preparations. The dyes were products of National Aniline Division of the Allied Chemical & Dye Corp. and were certified by the Biological Stain Commission.

All studies with fresh nuclei were made at pH 7.1 in the salt-sucrose solutions used for nuclear isolation (6).

Studies with the Feulgen reaction. Fresh rat liver nuclei (6) were fixed, washed, stained, and cleared in 15 ml centrifuge tubes. Routine staining procedures were used throughout. After each step the nuclei were sedimented centrifugally, the supernate discarded and replaced by the next solution. After dehydration through absolute alcohol the stained material was mounted on slides in Euparal or Diaphane.

To study the effects of heparin 0.8 ml of loosely packed nuclei and cells was made up to 2 ml with Solution III (6) and 6 mg of heparin in 1 ml of Solution 1 was added. After 8 minutes, when the characteristic viscous material had developed, it was either fixed for 15 minutes or hydrolyzed without prior fixation. The material was then sedimented, washed, stained, and mounted.

Several fixatives were employed. Acetic-alcohol was unsatisfactory in that it produced clumps of material which when hardened by dehydration made impossible the preparation of thin flat mounts. 10 per cent formalin (pH 3.4) was rather surprising in its action. When added to a suspension of cells or nuclei the suspension became somewhat viscous. It could, however, be sedimented in the clinical centrifuge. The prolonged washing necessary to remove the formaldehyde resulted in an appreciable loss of material. Good staining preparations were obtained from the residue. Formalin completely failed to coagulate or precipitate the viscous substance produced by the addition of heparin to the suspension of nuclei or cells, consequently no slides could be obtained by this method. Allen's B15 fixative produced satisfactory coagulation and resulted in a precipitate fine enough to permit the preparation of slides of sufficient thinness for study under oil immersion. Prolonged washing in 70 per cent alcohol with the addition of lithium carbonate to each wash was necessary to remove the color due to the fixative. In some cases nuclei were stained by routine Feulgen methods without prior fixation.

Coleman's modification of the Feulgen reagent was used and resulted in a colorless solution of excellent staining quality. Optimum staining was obtained after 10 min. hydrolysis at 60° and one hour staining time. Allowing the stained material to stand too long (overnight) in alcohol resulted in a diffusion of color from the nuclei into the cytoplasm. Routine dehydration times avoided this diffusion.

OBSERVATIONS

The effect of heparin and basic dyes on fresh nuclei. Fresh unfixed nuclei when treated with heparin were observed to fade until their outline was hardly visible. The separation of nuclei previously clumped gave evidence of extruded material sufficiently viscous to push the nuclei apart (2). Many

whole cells in the heparin treated preparations exhibited a clear perinuclear space seemingly free of granular material.

Nuclei treated with azure A (7) or zinc free methylene blue (dye concentrations 1:1000 in Solution I used for nuclear isolation) shrank rapidly, and in the presence of high concentrations of the dye stained very deeply. Methyl green in the same concentration did not stain the nuclei deeply and caused only slight shrinkage. No methyl green staining material comparable to the nuclear contents was seen outside the nuclei. The nuclear shrinkage with these dyes was very rapid indicating that the dyes penetrated easily.

Heparin reversed the effects of the dyes. The nuclei were observed to swell and fade rapidly. Methyl green and azure A staining material was observed around the nuclei. In nuclei very deeply stained with methylene blue the material extruded in response to heparin was often observed to be in the form of darkly stained rods forced out through openings on the surface of the nuclei (Fig. 6). The extruded rods exhibited a slight birefringence.

Similar shrinkage with azure A and subsequent swelling and release of stained material in response to heparin are shown in Figs. 4 and 5.

Figs. 1, 2, and 3 illustrate the same group of nuclei before treatment, after methylene blue (1:1000), and after heparin (1:500). The average volume of the methylene blue treated nucleus is about $\frac{1}{4}$ that of the original nucleus, while the same nucleus after dye and heparin treatment is approximately $\frac{5}{4}$ of the original size.

Observations with the Feulgen stain. As previously mentioned, the suspensions were deliberately designed to contain both nuclei and cells and do not represent the highest degree of purity which may be prepared by the nuclear isolation technique used (6).

The general picture of the normal (unheparinized) stained material is shown in Figs. 15–18. Most isolated nuclei are circular in outline with smooth margins. The internal picture varies slightly with the fixation employed.

Fig. 1. Crude fresh rat liver nuclear preparation in salt-sucrose solution (Solution I).

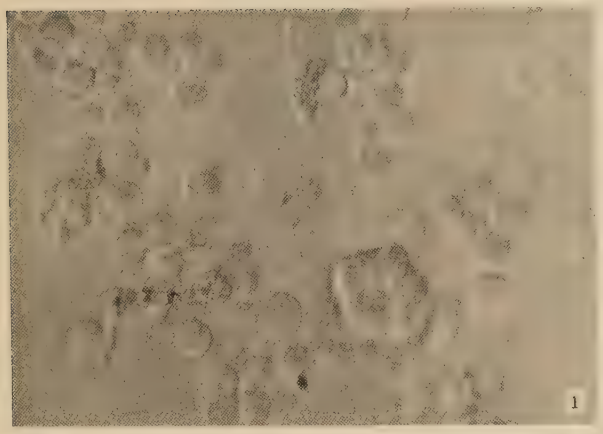
Fig. 2. Nuclei shown in Fig. 1 after treatment with zinc free methylene blue (1:1000) in solution I. Nuclei have shrunken to approximately $\frac{1}{4}$ their original volume. Few surface deformations are evident.

Fig. 3. Same nuclei as shown in Figs. 1 and 2 after treatment with heparin in solution I. Note extruded stained material on surface of nuclei. Much of the stain previously in the nuclei is now diffusely spread.

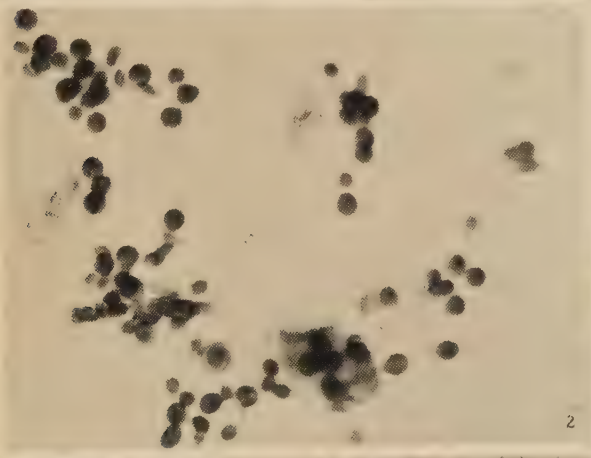
Fig. 4. Preparation of fresh rat liver nuclei after treatment with azure A (1:1000) in solution I. Little evidence of wrinkling of nuclear surface can be seen.

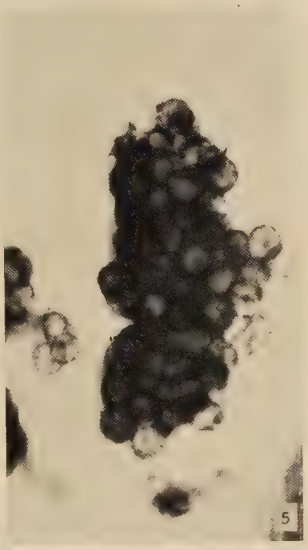
Fig. 5. Same nuclei as shown in Fig. 4 after treatment with heparin (1:500) in solution I. Azure A stained material could be observed to leave the nuclei.

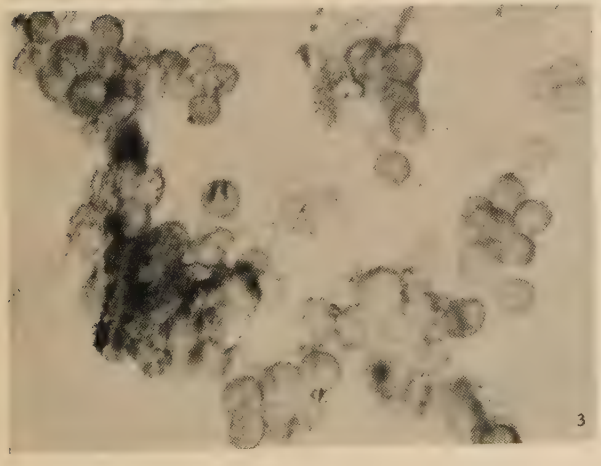
Fig. 6. Isolated nucleus treated with heparin after maximal shrinkage with methylene blue. Note rods of extruded stained material.

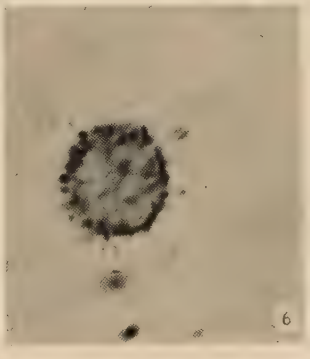

1

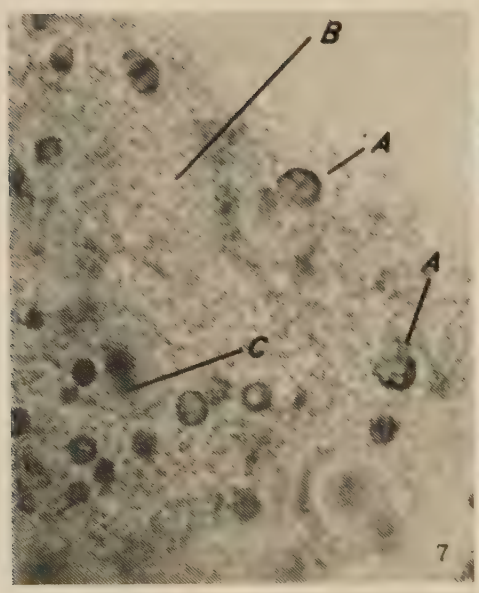
4

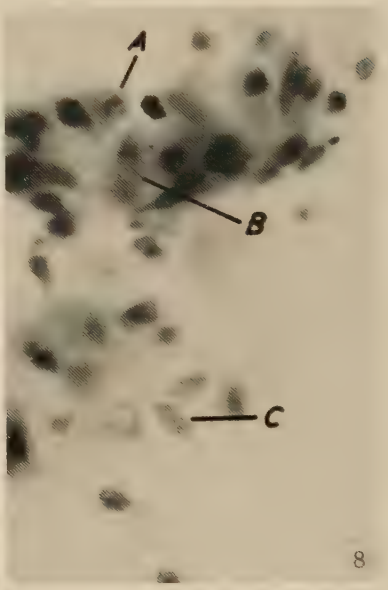

2


5


3

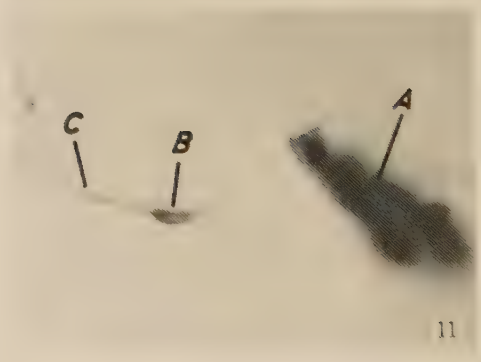

6

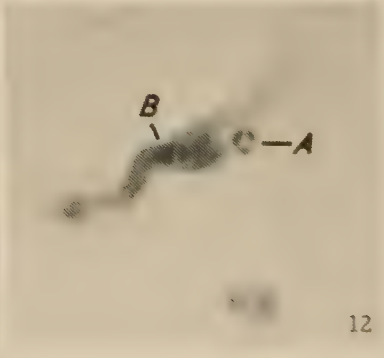

B
A
A
C
7


A
B
C
8

A
B
9

A
C
10


C
B
A
11


B
A
12

The Feulgen positive material is slightly more granular after B15 fixation than after formalin, but is uniformly distributed in both. Nucleoli show as faint outlines, lighter in color and presumably Feulgen negative, but this could not be positively ascertained in whole nuclei. In the cells present the cytoplasm appeared finely granular and totally devoid of stain. The nuclei of these cells were similar to the isolated nuclei.

About one nucleus in five showed some irregularity in outline with evidence of damage to or rupture of the nuclear membrane. Usually this damage was slight but in some cases nuclei were partially disintegrated and sometimes pulled out into thick threads (Fig. 15). A small amount of Feulgen positive debris, apparently representing nuclear fragments, was present (Fig. 18). Feulgen negative coagulum was present in fairly large quantity and recognizable denucleated cells were frequently seen. A thin film of cytoplasm was adherent to many isolated nuclei. Internal vacuolization of nuclei was rarely observed.

White blood cells were a constant feature of these preparations (Fig. 16). Approximately one nucleus out of each hundred was that of a w.b.c. Although some w.b.c. showed evidence of damage most appeared normal, with no evidence of structural damage and with visually intact cell membranes.

Heparin treated nuclei stained well and exhibited varying degrees of change attributable to heparin. Practically all nuclei showed changes, marked irregularity in outline, shape distortions, and varying degrees of internal vacuolization (Figs. 13 14). Surprisingly, in view of the large quantity of viscous material shown to contain DNA by chemical analysis (2), there was only slight evidence of the presence of Feulgen positive material

---

Fig. 7. Heparin treated, no fixation, Feulgen stain. Characteristic nuclear damage may be seen at A and in other nuclei. The coagulum, B, is Feulgen positive. A whole cell, C, contains Feulgen positive material in the cytoplasm. Some nuclei however show little sign of damage.

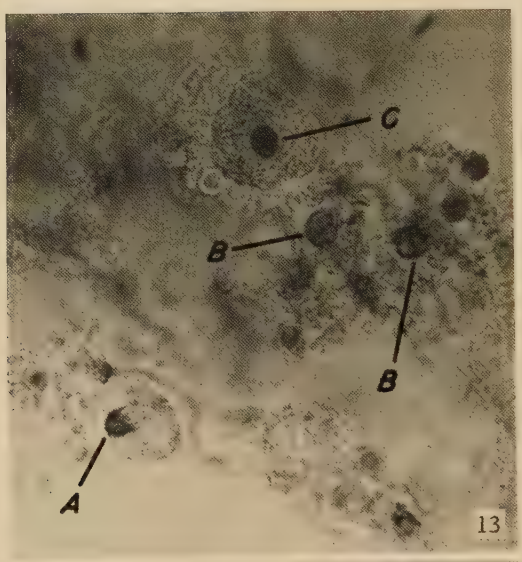
Fig. 8. Heparin treated, no fixation, Feulgen stain. A white blood cell showing no visible effects of heparin is seen at A. The coagulum, B, is Feulgen positive. The liver nucleus at C shows heparin damage, extreme vacuolization, and pale staining.

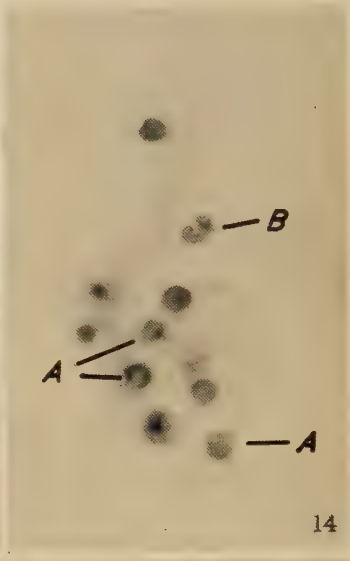
Fig. 9. Heparin treated, no fixation. Feulgen stain. Characteristic fibrous Feulgen positive coagulum is seen at A. An apparently undamaged white blood cell is seen at B.

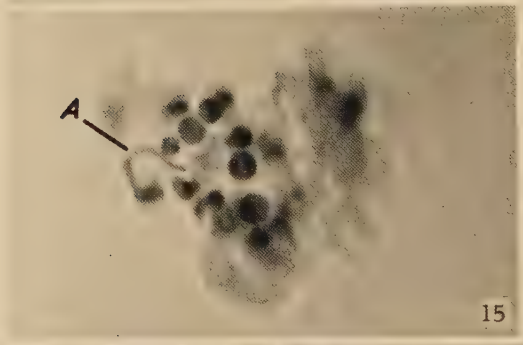
Fig. 10. Heparin treated, no fixation, Feulgen stain. Whole cell A contains damaged nucleus. Feulgen positive material within the cytoplasm is present at C.

Fig. 11. Heparin treated, no fixation, Feulgen stain. Characteristic isolated liver cell nucleus after heparin treatment is seen at B with accompanying fibrous Feulgen positive material C. A dense mass of Feulgen positive coagulum with entrapped nuclei visible at A.

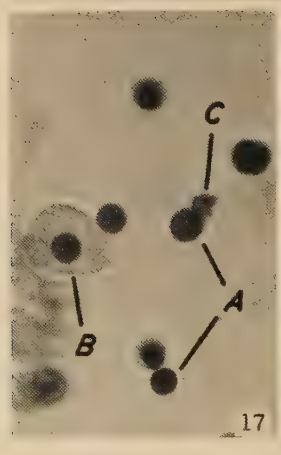
Fig. 12. Heparin treated, no fixation, Feulgen stain. Strongly Feulgen positive coagulum containing damaged nuclei at B, with adherent apparently undamaged white blood cell at A.

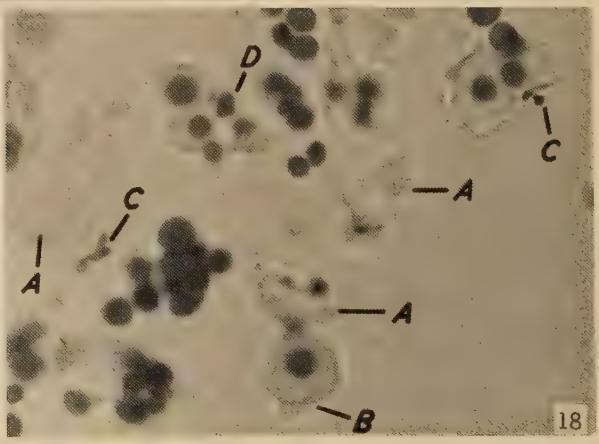

C
B
B
A
13


B
A
A
14


A
15

A
16


C
A
B
17


D
C
A
C
A
A
B
18

outside the nuclei. Large clumps of material gave a faint Feulgen test but this was seldom seen in isolated bits of tissue and coagulum. Three additional tests were performed with the quantity of heparin increased to 28 mg per 1.25 ml of packed nuclei and cells, and hydrolyzed between 6 and 20 min. In each case it was difficult to establish positively the presence of Feulgen positive material outside the nucleus.

In an effort to demonstrate more clearly the extra-nuclear DNA known to be present, resort was made to staining without prior fixation. Control (non-heparinized) nuclei were hydrolyzed without fixation and stained in the usual way. These slides gave the same general picture already described. It is noteworthy that a more intense stain was always obtained with the unfixed material than with any of the three fixatives tried. Heparinized nuclei were prepared as already described (0.8 ml nuclei and 6 mg of heparin) and hydrolyzed and stained without fixation. Figs. 7–12 show the effects of this treatment. Staining was intense and revealed the presence of large amounts of extranuclear Feulgen positive material in the form of fibers, strands, and thick masses. Nuclear damage was great, with marked vacuolization.

The nuclei of the white blood cells present did not differ in appearance from those seen in the control preparations. Damaged w.b.c. were seen in control preparations but were not seen in heparin treated material. Liver nuclei present in isolated whole cells and completely enclosed by cytoplasm were frequently as drastically affected as isolated nuclei (Fig. 13). Since the whole liver cells observed constantly showed irrregular margins, frequently with gross tears, the cell membranes cannot be presumed to be intact.

Fig. 13. Heparin treated, Allen's B15 fixation, Feulgen stain. Heparin damaged nucleus within whole cell at A. Other damaged nuclei are seen at B. The nucleus within a whole cell at C appears normal. Cytoplasm and coagulum appear to be very faintly Feulgen positive.

Fig. 14. Heparin treated, Allen's B15 fixation, Feulgen stain. Characteristic nuclear damage seen at A. The white blood cell at B does not show heparin damage. The fibrous coagulum appears Feulgen negative.

Fig. 15. No heparin, formalin fixation, Feulgen stain. Distorted nucleus A is pulled out into a Feulgen positive thread. Other nuclei when in focus appear normal.

Fig. 16. No heparin, no fixation, Feulgen stain. Normal nuclei are shown. A somewhat out of focus white blood cell is seen at A.

Fig. 17. No heparin, formalin fixation, Feulgen stain. Normal nuclei are seen at A and in other areas of the photograph. A whole cell is shown at B. The cytoplasm is colorless. Feulgen positive debris, presumably a nuclear fragment, is seen at C.

Fig. 18. No heparin, no fixation, Feulgen stain. Cytoplasmic debris, Feulgen negative, is seen at A. A whole cell showing damage to the cell membrane is seen at B. Feulgen positive debris appears at C. Most nuclei appear normal but occasional slight distortions may be seen at D. and elsewhere.

An interesting difference in the effect of the preliminary acid hydrolysis on unfixed heparinized and non-heparinized samples was noted. The supernate of the heparinized sample remained clear while the supernate of the non-heparin treated sample became cloudy. High speed centrifugation failed to sediment this cloudy supernatant material. The addition of heparin to the cloudy supernate resulted in the formation of a fine white easily sedimented precipitate which proved to be Feulgen negative. It could also be precipitated by ether-alcohol and gave the acid-mercuric chloride test for histone (11) and a positive reaction with a micro-biuret test (10). The supernate of the heparin-treated preparation which was also Feulgen negative failed to give a positive test for histone or protein and gave no precipitate on the addition of heparin.

DISCUSSION

In attempting to confirm the presence of DNA in the heparin produced extra-nuclear gel, use was made of azure A, methyl green, and methylene blue staining. Positive tests were obtained with all three on the extruded gel. However it was noted that pretreatment of the nuclei with these stains (1 : 1000 in a sucrose-phosphate buffer) resulted in a sudden drastic shrinkage in the case of azure A and methylene blue, and a slight shrinkage with methyl green. These effects were reversed when heparin was introduced in the same buffer, the nuclei promptly swelling to more than their original size. Stained material could now be identified outside the faded nuclei. These results are taken as additional confirmation of the release of DNA by heparin from the isolated nucleus (6).

Nuclei maximally stained with methylene blue when treated with heparin were observed to extrude rods of stained material through apertures scattered over the nuclear surface. The birefringence observed in these rods suggests some degree of orientation of DNA molecules in the extruded material. It should be noted that the existence of regularly spaced apertures in the surface of the immature frogoocyte nucleus has been previously reported (8).

No wrinkling was evident in the rapidly shrinking nuclei until maximal shrinkage had been obtained, at which time slight irregularities were occasionally observed. The surface of nuclei isolated by the procedure used here (6) seems therefore to possess very elastic properties.

The rapid shrinkage in the presence of positively charged dyes and the rapid swelling in the presence of the negatively charged heparin molecules are characteristic of the behavior of negatively charged gels.

It has been pointed out previously (6, 2) and should be emphasized again that there is no way of determining at this time what changes may have

occurred in nuclear ultrastructure during the isolation procedure. No definite conclusions can therefore be made as to whether the observations on isolated nuclei are directly applicable to intact intracellular nuclei.

As further evidence of the nucleic acid releasing action of heparin on isolated liver nuclei, the presence of Feulgen positive material outside the nuclei has been shown. No final conclusions can be drawn from these observations but they invite and provide some basis for speculation as to the mode of action of heparin on cell components.

It has been noted that although isolated liver nuclei and those within liver cells show characteristic heparin damage, the nuclei of white blood cells whose cell membranes were visually intact showed no such damage. There seems to be no reason to assume a chemical difference in leucocyte nuclei to account for this resistance. Liver nuclei completely enclosed in cytoplasm were not protected, but in these cells the cell membrane was clearly not intact. This suggests that with the heparin concentration used either the intact cell membrane is not permeable to heparin or that more nearly normal cells (leucocytes) are not as subject to heparin damage as are injured cells.

Acid hydrolysis of fresh tissues has been used to free them of histone (9) and such hydrolysis was necessary in these studies to demonstrate the presence of extranuclear Feulgen positive material after heparin treatment. When freshly prepared suspensions of nuclei which did not contain heparin were subjected to acid hydrolysis as in routine Feulgen procedure, a cloudy non-sedimentable supernate resulted which was precipitated by heparin and gave positive protein (biuret) and histone (acid-mercuric chloride) tests. The supernate of hydrolyzed heparin treated suspensions remained clear, formed no precipitate with heparin and failed to give positive protein and histone tests. This suggests that the action of heparin may be the replacement of DNA in the nucleohistone of the nucleus by heparin, resulting in the structural damage noted, the release of DNA from the nucleus, and its subsequent partial combination with other protein, to form the previously described (1, 2) viscous material.

It has not been possible to determine positively why fixed preparations failed to show extranuclear DNA. Washing is essential to remove the fixative, and washing without prior fixation introduces additional factors. Either fixing or washing or both may be responsible. However, the fact that DNA, displaced from the nuclei by heparin (a naturally occurring substance) fails to give a positive Feulgen test when the customary fixative are used, but does give a positive Feulgen reaction without fixation suggests that the test depends to some extent on the manner in which the DNA is bound. This suggests

the possibility that the Feulgen reaction as ordinarily used may indicate some part of the nuclear DNA which may or may not be a constant fraction of the total DNA.

SUMMARY

The gel released from isolated rat liver nuclei in response to heparin treatment has been found to stain with methylene blue, azure A, and methyl green when the dyes were added to the salt-sucrose nuclear isolation medium.

Azure A and methylene blue caused rapid nuclear shrinkage to as little as $\frac{1}{4}$ the original nuclear volume. Subsequent treatment with heparin caused the nuclei to fade rapidly and swell to approximately $\frac{5}{4}$ of the original volume. With methylene blue stained nuclei heparin caused the extrusion of deeply stained, slightly birefringent rods through apertures on the nuclear surface. Methyl green also caused nuclear shrinkage, but to a lesser degree.

Studies with the Feulgen reaction demonstrated structural damage in isolated rat liver nuclei as a result of heparin action. The viscous material released by heparin was shown to be Feulgen positive by resort to hydrolysis without prior fixation, since after customary fixatives the presence of a Feulgen positive reaction outside the nucleus could not be clearly demonstrated. The possibility is suggested that the Feulgen reaction following the customary fixatives depends in part on the manner in which the DNA is bound.

The nuclei of leucocytes with visually intact cell membranes included in the nuclear preparations failed to show structural damage due to heparin and it is suggested that either the cell membrane provides some protection against heparin action or that damaged cells are more susceptible to this action.

Observations made provide additional basis for the conclusion that heparin replaces DNA in the nucleo-histone of the nucleus, resulting in the structural damage observed, and releasing DNA in the form of a soluble viscous protein containing complex.

REFERENCES

1. Anderson, N. G., and Wilbur, K. M., *Fed. Proc.*, **9**, 254 (1950).
2. —— in press.
3. Heilbrunn, L. V., and Wilson, W. L., *Proc. Soc. Exp. Biol. Med.*, **70**, 179 (1949).
4. —— *Science*, **112**, 56 (1950).
5. Allen, J. G., and Jacobsen, L. O., *Science*, **105**, 388 (1947).
6. Wilbur, K. M., and Anderson, N. G., *Exp. Cell Res.*, **2**, 46 (1951).
7. Flax, M., and Pollister, A. W., *Anat. Rec.*, **105**, 536 (1949).
8. Callan, H. G., Randall, J. T., and Tomlin, S. G., *Nature*, **163**, 280 (1949).
9. Kurnick, N. B., *Exp. Cell Res.*, **1**, 151 (1950).
10. Weichselbaum, T. E., *Am. J. Clin. Path., Techn. Sect.*, **10**, 40 (1946).
11. Mirsky, A. E., and Pollister, A. W., *J. Gen. Physiol.*, **30**, 117 (1946).

SYNTHESIS OF NUCLEIC ACID PURINES IN THE SEA URCHIN EMBRYO

RICHARD ABRAMS[1]

Institute of Radiobiology and Biophysics, University of Chicago, Chicago, Ill. and the Marine Biological Laboratory, Woods Hole, Mass.

Received September 13, 1950

The virgin egg of the sea urchin, *Arbacia punctulata*, contains relatively large amounts of pentosenucleic acid (RNA) but only traces of desoxypentosenucleic acid (DNA). As the embryo develops after fertilization, the amount of DNA increases rapidly as was shown by Brachet (3, 4). Brachet also observed that the RNA content decreased to the same extent that DNA increased, in agreement with the earlier findings of Masing (11) and of Needham and Needham (12) that the total amount of nucleic acid purine and phosphorus remained constant during early development. On the basis of these observations Brachet postulated that RNA served as the precursor of DNA (4). He termed this "partial synthesis" of DNA in contrast to "total synthesis" in avian embryos where the virgin egg has only traces of either nucleic acid.

Schmidt, Hecht, and Thannhauser (13) repeated Brachet's work, using as an analytical method the phosphate fractionation devised by Schmidt and Thannhauser (14). Their results did not confirm the "partial synthesis" theory in that, while DNA increased some 18 fold, RNA remained constant during the first 24 hours of development. Villee *et al* (16) repeated these experiments and found qualitatively the same results—the RNA content remained constant for 20 hours after fertilization while DNA increased 6 fold. Thus there exists a conflict between the results obtained by phosphate fractionation which indicated an increase of some 50 per cent in total nucleic acid during 24 hours of development and the earlier analysis for total phosphorus and total purine and for pentose and desoxypentose which indicated that total nucleic acid content remained more or less constant. It must be

[1] This work was aided by a Fellowship grant from the Atomic Energy Commission for work at the Marine Biological Laboratory during the summers of 1948 and 1949.

pointed out that the results obtained by the Schmidt-Thannhauser method cannot be accepted without question. Furst, Roll, and Brown (8) have pointed out that this method results in 4 per cent of the RNA appearing in the DNA fraction. Thus the values obtained for DNA content of virgin eggs probably represent nothing but RNA contamination. Indeed, the observation of Schmidt *et al* that the DNA content of the sperm was only 3 per cent of that of the egg probably indicated that 97 per cent of the supposed DNA of the egg was RNA. RNA contamination may also account for the observation of Villee *et al* that no significant DNA increase occurred during the first 6 hours of development, despite the fact that the number of cells (and of nuclei) increased by several orders of magnitude. In addition, phosphate fractionation must be checked by other methods to eliminate the possibility that other acid-insoluble phosphate compounds, such as metaphosphates for example, are found in sea urchin eggs.

A more definitive method of investigating the concept of "partial synthesis" involves the use of isotopic tracers and is the subject of this paper. If a nucleic acid precursor is administered to the developing embryos, and if RNA is a precursor of DNA, then the specific activity of DNA can never exceed that of its precursor, RNA. While the present work was in progress, Villee *et al* (16) carried out such experiments with P^{32} using the Schmidt-Thannhauser fractionation and found that the specific activity of DNA-P was 2 to 15 times that of the RNA-P fraction indicating that RNA could not have been the direct presursor of DNA. In view of the difficulties mentioned above in the phosphate method and the known difficulty of removing traces of adsorbed P^{32} from crude tissue fractions, the present work was carried out with the purine precursor, glycine-1-C^{14}, and pure guanine and adenine isolated. The results obtained agree with those of Villee in demonstrating that the DNA at the blastula stage had more than 10 times the specific activity of the RNA. The larger part of the DNA purines, however, probably were derived from an endogenous precursor the nature of which is unknown at present.

EXPERIMENTAL

Incubation of Fertilized Eggs with Labeled Substrate. Eggs were obtained from *Arbacia* by the following procedure. The oral surface was cut away and the urchin set in a bowl of filtered sea water. After touching the gonads lightly with a finger, eggs were exuded through the gonopores. Pooled eggs from 30 to 40 females were washed 4 times by decantation with filtered sea water, and then fertilized by the addition of a few drops of dilute sperm suspension. The yield of eggs averaged 50 mg dry weight per animal. Over 90 per cent of the eggs fertilized normally.

After fertilization, the embryos were allowed to develop in 500 ml sea water containing the labeled substrate in a concentration of 0.001 M to 0.01 M. In the early experiments, numbered 48, the substrates were glycine-1-C^{13} and acetate-l-C^{14}. Incubation was carried out in open dishes with periodic stirring for periods up to 24 hours. It was found that the dense suspensions used could not obtain enough oxygen under these conditions—development proceeded uniformly through 3 or 4 cleavages but by 10 hours only $\frac{1}{8}$ to $\frac{1}{2}$ had reached the blastula stage and there was no further development. This difficulty was eliminated in the later experiments, numbered 49, with glycine-1-C^{14}. The egg suspension was placed in a 2.8 liter Fernbach flask through which a slow stream of oxygen bubbled. The flask was set on a rotary action shaking device so that the suspension was continuously agitated with a slow swirling motion. The oxygen stream served to aerate the suspension and carry off any radioactive carbon dioxide which was then trapped as barium carbonate. After 12 hours the embryos were present as free-swimming blastulae.

At the end of the incubation period the embryos were centrifuged lightly and washed 3 times with 100 ml portions of absolute alcohol. An alcohol suspension was vibrated for 6 hours with glass beads (60 cycles/second) to completely fragment the cells. The debris was suspended in 150 ml of an alcohol-ether mixture (3:1) and refluxed for 2 hours to remove the lipids, washed with ether, and air dried. The dry powder was used for nucleic acid or purine isolation.

Chemical Fractionation. In the experiments numbered 48 the combined nucleic acids were isolated in pure form by the method of Hammarsten (9). For 1 to 2 g of embryo powder this involved 8 extractions with 40 ml portions of Hammarsten's salt-urea mixture, precipitation as the cupric salt, precipitation as the potassium salt from alcohol, and finally precipitation as the free acid from 0.1 M HCl in absolute alcohol at —18°. This cycle of extractions and precipitations was repeated 3 times before a white, protein-free product was obtained. The average yield of mixed nucleic acid was 4 per cent of the dry weight. The DNA and RNA were separated by the uranyl acetate procedure described previously (2), i.e. 2 hour hydrolysis at 100° in 0.1 M NaOH followed by precipitation of the DNA with an uranyl acetate-trichloroacetic acid mixture. The uranyl precipitate was twice dissolved in 0.1 M NaOH and reprecipitated. The supernatant solution containing RNA was adjusted to pH 4.65 to precipitate the uranyl mononucleotides of RNA, and these were dissolved in acid and reprecipitated 3 times. The nucleic acids were then combusted for isotope analysis without further fractionation.

Direct isolation was not used in the experiments numbered 49. Instead, the tissue was separated into RNA and DNA fractions by a modification of the method of Schmidt and Thannhauser (14) as described in detail for mammalian tissues elsewhere (2). Acid soluble purines were first removed by dissolving the lipid-free powder in 0.1 M NaOH at 0° and immediately reprecipitating with a mixture of 0.11 volumes of 1 M HCl and 0.12 volumes 50 per cent trichloroacetic acid. The precipitate was washed repeatedly with 5 per cent trichloroacetic acid at 0°, then with alcohol and ether and air dried. This was kept in 0.1 M NaOH at 100° for 2 hours to hydrolyze the RNA, and DNA was then precipitated with the hydrochloric-trichloroacetic acid mixture as before. The supernatant solution was taken as the RNA fraction. The DNA precipitate was twice reprecipitated to remove traces of RNA, then extracted with 5 per cent trichloroacetic acid at 100° for 20 minutes to hydrolyze the

DNA (15). The extract was taken as the DNA fraction, the residue as the protein fraction. Purines were liberated from both RNA and DNA fractions by adding concentrated hydrochloric acid to make the final concentration 1 M, and refluxing for 1 hour at 100°. An excess of ammonium hydroxide and 1 M silver nitrate were added to precipitate the purines as silver salts. The latter were decomposed with 1 M hydrochloric acid, and guanine and adenine separated in pure form by cation exchange chromatography as previously described (1).

Measurement of Radioactivity. All samples were combusted to carbon dioxide which was precipitated as barium carbonate. The specific activity was taken as the observed counts per minute in a windowless flow counter when the barium carbonate was spread as an infinitely thick layer ($\sim$20 mg/cm^2) in an aluminium dish of 1.41 cm^2 area. The C^{13} determinations were made in a Consolidated-Nier isotope ratio mass spectrometer.

RESULTS AND DISCUSSION

In Table I are summarized the results of a preliminary group of experiments designed to test whether the carbon skeleton of the nucleic acids was synthesized from the small molecules, acetate-1-C^{14} and glycine-1-C^{13}, in a fashion analogous to the phosphate utilization demonstrated by Villee *et al* (16). In both experiments the incubation time was 20 hours, although no observable development took place after 12 hours. In number 48–3 the stirring was more vigorous and development more uniform to the early blastula stage. As with P^{32}, the specific activity of DNA was found to be 10 to 14 times greater than that of RNA.

At the time these experiments were carried out (Summer 1948) it was believed that acetate was a precursor of the 2 and 8 carbon atoms of purine as reported by Buchanan, Sonne, and Delluva (6) who had also demonstrated that the carboxyl group of glycine was the precursor of carbon atom 4. This result was apparently erroneous and it is now believed that only small amounts of acetate carboxyl carbon appear in both purines and pyrimidines (10) as a result of a preliminary conversion to carbon dioxide. We have observed in unpublished experiments that the specific activity of purines from acetate-labeled mammalian nucleic acid is slightly less than that of the total nucleic acid. It would seem then, that C^{14} administered as acetate-1-C^{14} appears to some extent in purine, pyrimidine, and sugar of the nucleic acid. Thus, from the data in Table I and the observations of Villee *et al* with P^{32}, one can conclude that the specific activity of DNA was considerably greater than that of RNA not only in the purines and phosphate but probably in other parts of the molecule as well.

The results of these experiments prove that DNA was not synthesized

TABLE I

Incorporation of Labeled Acetate and Glycine in Nucleic Acids and Protein of the Sea Urchin.

Experiment Number	48–2	48–3	
Concentration of labeled substrates	0.001 M	0.001 M	
	Specific Activity counts/min.	Specific Activity counts/min.	C^{13} Concentration atom % excess
Acetate-1-C^{14}, carboxyl group	2.6×10^5	2.6×10^5	—
Glycine-1-C^{13}, carboxyl group	—	—	50.9
DNA, total	155	835	0.488
RNA, total	15	65	0.035
Protein, total	30	—	—
Protein, α carboxyl	—	535	0.224
DNA/RNA specific activity ratio	10.3	12.8	13.9

solely from pre-existing RNA as proposed by Brachet. However, the possibility still exists, as discussed by Villee, that a very small fraction of the RNA of the order of a few per cent which is chemically distinct or physically isolated from the bulk of the RNA may have the properties of being synthesized very rapidly and serving as a precursor for DNA. Proof of this possibility must await the demonstration that sea urchin RNA can be fractionated into more than one moiety.

Another possibility which was not considered in the experiments of Villee referred to in the preceding paragraphs was the possibility that only a trace of the newly formed DNA arose from the labeled precursor while the vast bulk was formed from some endogenous precursor, as RNA for example. This can be tested by comparing the specific activity of the labeled precursor with the specific activity of the carbon atoms in the nucleic acid in which it appears. Since virgin eggs have only traces of DNA, the DNA in embryos by the time they have reached the blastula stage must all be newly formed, and the specific activity of the carbon atoms in position 4 of the DNA purines must have the same specific activity as the carboxyl group of the glycine-1-C^{14} in the medium, provided that no endogenous source of purines exists.

A group of experiments, summarized in Table II, was carried out to measure quantitatively the dilution of the precursor carbon by isolating the purines in pure form. In experiment 49–4 the embryos were grown for 6 hours in 0.01 M glycine-1-C^{14}, at which time they were transferred to a fresh glycine-1-C^{14} solution and grown for 6 more hours. Experiments 49–6 and

Table II

Formation of Nucleic Acid Purines from Glycine-1-C^{14} in the Sea Urchin.

Experiment Number	49–4	49–6	49–62
Glycine concentration	0.01 M	0.005 M	0.005 M
	Specific Activity counts/min.	Specific Activity counts/min.	Specific Activity counts/min.
Glycine-1-C^{14}, carboxyl group	0.95×10^5	1.83×10^5	1.30×10^5
DNA, adenine	3 290	3 420	2 380
DNA, guanine	6 900	9 220	4 850
RNA, adenine	401	525	492
RNA, guanine	376	608	443
Respiratory CO_2	7 310	13 600	11 600

49–62 were identical except that no glycine was added until after 6 hours had elapsed, and the embryos were then grown in 0.005 M glycine-1-C^{14} for the second 6 hour period. At the end of this 12 hour period the embryos had reached the free-swimming blastula stage.

In confirmation of the previous experiments, the specific activities of the DNA purines were greater by an order of magnitude than those of the RNA purines. The two purines, adenine and guanine, of RNA had essentially the same specific activity which was to be expected if they were formed from glycine through a common precursor or if all the intermediate precursors were diluted by endogenous precursor to exactly the same extent. With DNA, however, guanine always had more than twice the specific activity of adenine. In this case, at least, adenine could not have been a precursor of guanine as has been suggested for rat nucleic acids by Brown, Roll, Plentl, and Cavalieri (5).

In order to compare isotope dilutions, the C^{14} concentrations in carbon atom 4 of the purines have been calculated in Table III relative to the glycine carboxyl group taken as 100. It is obvious that in no case did the specific activity approach 100. A value less than 100 would result if the C^{14} of the added glycine were diluted by "metabolic pool" glycine of the organism, or if part of the purine were formed from an endogenous precursor other than glycine. Although direct measurements of glycine dilution were not made, the fact that varying the concentration from 0.001 M to 0.01 M had almost no effect on the extent of incorporation in the purines indicated that glycine dilution was not an important factor. One must then conclude that 75 per

TABLE III

Specific Activities of Nucleic Acid Purines Relative to the Labeled Precursor (Glycine-1-C^{14}).

Experiment Number	49–4		49–6		49–62		48–3	
Glycine concentration	0.01 M		0.005 M		0.005 M		0.001 M	
	Relative specific activity compared to the carboxyl group of glycine taken as 100[1]							
	Guanine	Adenine	Guanine	Adenine	Guanine	Adenine	Guanine	Adenine
RNA	1.97	2.10	1.66	1.43	1.70	1.89	—	—
DNA	36.2	17.2	25.2	9.35	18.7	9.15	(28.5)[2]	(12.4)[2]

cent of the DNA guanine and 88 per cent of the DNA adenine were not synthesized from labeled glycine but arose from other endogenous precursors. At the present time one can only speculate as to the nature of this endogenous source of purines. In the complex series of reactions from glycine to nucleic acid purine, dilution might have taken place at any one of the many steps. One possible source of purine diluent was the RNA, but any simple conversion mechanism of RNA or its hydrolysis products to DNA is ruled out by the fact that DNA adenine had less than half the specific activity of DNA guanine while the two purines had the same specific activity in RNA. Several explanations are possible based either upon differential enzyme activity or varying permeability of intracellular membranes but such speculation must await further experimental evidence.

One can only conclude at present that the hypothesis that newly formed DNA must arise from the RNA originally present in the virgin egg is incorrect because, in the experiments reported here, 12 per cent of the DNA adenine and 27 per cent of the DNA guanine were newly formed from glycine as compared to approximately 1.8 per cent turnover of RNA purines relative to glycine. However, the remainder and larger part of the DNA purines must have arisen from some endogenous source which may or may not have been RNA.

[1] In making these calculations, the observed specific activities of the purines were multiplied by 5 since only one of the five carbons (that in position 4) of the purine is derived from the glycine carboxyl group (2, 6).

[2] These values were calculated from the observed specific activity of the total nucleic acid by assuming (a) that only the carbon atoms in position 4 of the purines were labeled; (b) that sea urchin DNA has the composition reported by Daly, Allfrey, and Mirsky (7); and (c) that the adenine/guanine specific activity ratio was the average of those obtained in experiments 49.

ACKNOWLEDGEMENT

The author is indebted for technical assistance in carrying out the analyses to Miss Amelia Cherkes and in synthesizing labeled compounds to Mr. Leon Clark.

SUMMARY

Embryos of the sea urchin, *Arbacia punctulata*, were shown to utilize carboxyl labeled acetate and glycine for the synthesis of nucleic acids and protein.

The hypothesis that DNA must arise exclusively from the pre-formed RNA of the virgin egg is unlikely since the specific activities of DNA and its isolated purines from the blastula were at least 10 times greater than the corresponding values for RNA.

The specific activities of adenine and guanine of RNA formed from glycine-1-C^{14} were identical, whereas in DNA the specific activity of adenine was less than half that of guanine.

Comparison of the specific activities of the DNA purines with the glycine in the medium indicated that a large part of the newly formed DNA arose from unlabeled endogenous precursor.

REFERENCES

1. Abrams, R., *Arch. Biochem.* (in press).
2. —— *Arch. Biochem.* (in press).
3. Brachet, J., *Arch. Biol. Liège*, **48**, 529 (1937).
4. —— Embryologie Chimique, Masson & Cie, Paris, 1947, p. 208.
5. Brown, G. B., Roll, P. M., Plentl, A. A., and Cavalieri, L. F., *J. Biol. Chem.*, **172**, 469 (1948).
6. Buchanan, J. M., Sonne, J. C., and Delluva, A. M., *J. Biol. Chem.*, **173**, 81 (1948).
7. Daly, M. M., Allfrey, V. G., and Mirsky, A. E., *J. Gen. Physiol.*, **33**, 497 (1950).
8. Furst, S. S., Roll, P. M., and Brown, G. B., *J. Biol. Chem.*, **183**, 251 (1950).
9. Hammarsten, E., *Acta Med. Scand., Suppl.*, **196**, 634 (1947).
10. Heinrich, M. R., Wilson, D. W., and Gurin, S., *Am. J. Med. Sci.*, **217**, 709 (1949).
11. Masing, E., *Z. physiol. Chem.*, **67**, 161 (1910).
12. Needham, J., and Needham, D. M., *Brit. J. Exp. Biol.*, **7**, 317 (1930).
13. Schmidt, G., Hecht, L., and Thannhauser, S. J., *J. Gen. Physiol.*, **31**, 203 (1948).
14. Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, **161**, 83 (1945).
15. Schneider, W. C., *J. Biol. Chem.*, **161**, 293 (1945).
16. Villee, C. A., Lowens, M., Gordon, M., Leonard, E., and Rich, A., *J. Cellular Comp. Physiol.*, **33**, 93 (1949).

CHROMOSOME STRUCTURE IN ESCHERICHIA COLI

ALFRED MARSHAK

N. Y. U. College of Medicine and Division of Tuberculosis, U.S. Public Health Service, New York

Received Sept. 25, 1950

Bodies have been described in Escherichia coli, which are visible with the light microscope and show the characteristic response of nuclei toward stains and enzymes, and similar bodies have been reported in electron microscope studies (1, 2, 8, 9). However in none of these studies was the internal structure of the nucleus or chromosomes described. The observations here reported show the presence of structures resembling chromonemata in morphology, while chemical analyses of the particulate fraction in which the apparent nuclear bodies are concentrated fulfill expectations from results of previous investigations.

The bacteria were grown in one-liter batches in Fernbach flasks at 37° C. The medium, developed by Miss Jane Pinsent (personal communication), was the following:

KH_2PO_4	0.02 per cent
Ethylene diamine tetra-acetic acid	0.05 » »
$MgSO_4 \cdot 7H_2O$	0.08 » »
NH_4Cl	0.02 » »
$Na_2 \cdot$ succinate $\cdot 2H_2O$	0.2 » »
NaH · glutamate	0.3 » »
Ca (as $CaCl_2$)	2.0 mg per cent
Zn (as $ZnSO_4 \cdot 7H_2O$)	5.0 » » »
B (as H_3BO_3)	2.0 » » »
Mn (as $MnCl_2 \cdot 4H_2O$)	1.4 » » »
Fe (as $FeSO_4 \cdot 7H_2O$)	1.0 » » »
Mo (as $(NH_4)6Mo7O_{24} \cdot 4H_2O$)	0.6 » » »
Cu (as $CuSO_4 \cdot 5H_2O$)	0.6 » » »
Co (as $CoCl_2 \cdot 6H_2O$)	0.4 » » »

The pH was adjusted to 6.8 7.0 with KOH. The use of ethylene diamine tetra-acetic acid has previously been discussed (3.)

The 24-hour cultures were harvested by centrifugation, resuspended in 5–10 ml 0.85 per cent NaCl, and inoculated into fresh medium prewarmed to 37° C. After 2 hours, during which the bacteria doubled in number, they were again removed, suspended in 24 ml 0.85 per cent NaCl, and the suspensions made 5 per cent with respect to citric or acetic acid immediately before sonic treatment. Approximately 25 ml of the acidified bacterial suspension was placed in the water-cooled sample cup of a Raytheon 50 W, 9 Kc, magnetostriction oscillator (Model S 102). The maximum plate voltage (140 V) was applied and the frequency adjusted so that the output voltage was at a minimum (120–125 V). The bacteria were treated for 45 minutes, the shortest time required for obtaining 90–95 per cent destruction. After the sonic fragmentation, the suspension was considerably darkened. Such darkening did not occur when the bacteria were allowed to remain in 5 per cent citric or acetic acid for several days without sonic treatment, nor was it observed when either citric or acetic acid without bacteria were shaken in the oscillator.

The bacterial fragments were subjected to centrifugation in a Servall SS-2 vacuum angle centrifuge. At 900 g for 20 minutes the suspension was separated into a black sediment and a colorless supernatant. The sediment was repeatedly washed (4–5 times) until the supernatant fluid was almost water-clear. The combined supernatant fluids were then again centrifuged at 900 g to remove residual heavy particles carried over, and then subjected to 3,600 g for 30 minutes and the sediment so obtained, removed. Very little material visible in the phase-contrast microscope remained in the supernatant fluid. No visible difference was noticed when the light particles were collected at 55,000 g. The separation of the bacterial fragments into a heavy black fraction and a light colorless one was independent of the type of medium in which the bacteria were grown. Experiments in which the bacteria were grown in 1 or 5 per cent tryptose+0.5 per cent glycerol, or in Friedlein medium, gave results similar to those described above.

Under the phase-contrast microscope the heavy fraction was found to be a rather uniform suspension of black particles often clustered, but usually single (Fig. 1). The light fraction was a more heterogeneous mixture of particles of smaller average size (Fig. 2). On standing either at room temperature or at 2° C both types of particles aggregated into large clumps.

Air-dried or heat-fixed bacteria gave a satisfactory Feulgen stain i. e., in most of the bacteria there were one or two red bodies in a colorless background, usually central or subcentral but never polar. Bacteria fixed in citric or acetic acid even after thorough washing in distilled water showed only a diffuse pink stain or no stain at all. The heavy bacterial fragments, being dense black could not be stained. Occasionally in the Feulgen-stained preparations, red stained bodies of the size and shape of those observed on Feulgen staining of the air-dried bacteria, were found embedded in an amorphous matrix which presumably protected them against the black pigment. On the assumption that the black pigment might be an oxidation

product, bacteria were also subjected to sonic treatment in the citric acid solution to which 2 per cent ascorbic acid was added. However the heavy particles so obtained were just as black as ever.

The whole bacteria and the two particulate fractions were analysed for their purine and pyrimidine content. Bacteria collected from 6 liters of medium were used to prepare the particulate fractions. Each was extracted twice for 10 minutes in cold 5 per cent trichloracetic acid, then once with 70 per cent ethanol, twice with 95 per cent ethanol, and finally twice with a 2 : 1 mixture of boiling ethanol and ether. The solvents were removed by centrifugation and the sediments dried in a vacuum desiccator. The yields were 23.0 mg residue from the heavy fraction and 75.2 mg from the light fraction. Thirty mg of the dried materials were digested with 0.16 ml 70 per cent $HClO_4$ for 1 hour at 100° C (6, 7). The clarified digest diluted to 1 ml was then subjected to chromatographic separation on filter paper, using two solvent systems, 7 volumes n-butanol and 1 volume of N/10 NH_4OH to obtain guanine, cytosine, thymine, and a mixture of adenine and uracil; and 7 volumes of n-butanol plus 1 volume N HCl to obtain uracil and thymine. The adenine was obtained by difference (6).

The results are given in the following table:

	Nucleic Acid Bases (in μg/mg dry matter)				
	Adenine	Guanine	Cytosine	Uracil	Thymine
Intact bacteria	18.6	24.3	13.9	7.39	3.32
Heavy particles	10.6	15.3	12.1	4.46	2.34
Light particles	19.9	25.1	14.8	8.76	3.85

The small particle fraction had a somewhat higher base content than the whole bacteria, while that of the large particles was much lower, particularly in guanine, adenine, and uracil. The following table gives the molar proportions of the bases relative to adenine as unity:

	Molar ratios				
	Adenine	Guanine	Cytosine	Uracil	Thymine
Intact bacteria	1	1.21	0.94	0.50	0.20
Heavy particles	1	1.28	1.38	0.50	0.24
Light particles	1	1.13	0.91	0.53	0.21

In the large particle fraction there was a greater amount of cytosine and thymine relative to adenine than in the whole bacteria or the small particle fraction.

For electron microscopy, suspensions of whole bacteria and of the particulate fractions were diluted with distilled water and drops of these dilutions placed on collodion films mounted on glass microscope slides which were then placed on a block of solid CO_2 until the drops were frozen. They were then put into the chamber of the metal evaporating apparatus which was immediately evacuated. Chromium was used for shadowing. The shadowed film containing the biological material was then floated off in water and mounted on a supporting copper screen. Another method which gave better results was to immerse the supporting screen with its collodion film in the diluted suspensions which were then subjected to centrifugation at appropriate speeds. The screens were removed to strips of filter paper which were then put on solid CO_2 until the residual liquid on the film was frozen, after which it was shadowed with chromium. Using similar procedures, electron micrographs were also taken of unshadowed cells and particles.

Whole bacteria, shadowed shortly after being treated with citric or acetic acid, appeared to be uniformly dense in the electron micrographs which were similar to those described by Hillier, Mudd, and Smith (2). When the same bacteria were allowed to remain for three days in the acetic acid at 2° C and electron micrographs taken without shadowing, most bacteria were found to have oval areas of much lower density than the major portion of the cell. When viewed from the side, these areas were found to extend across $\frac{1}{2}$–$\frac{2}{3}$ of the short axis of the cell and about $\frac{1}{4}$ of the long axis. In face view these areas occupied the full width of the cell. In general, the appearance suggested that bacterial substance had been ejected from the cell as a solid body (Fig. 3). In one instance, in the neighborhood of what appeared to be a completely disrupted cell, some helically coiled structures were found (Fig. 4). No such helical structures were found in the unshadowed micrographs of the amorphous bacterial debris obtained by high speed centrifugation following sonic treatment and removal of the heavier particles.

When the heavy particulate fraction was shadowed with chromium, a number of particles were found to be more or less regularly lobulated at the periphery, suggesting the presence of either a banded or helical structure. At higher magnifications, the lobulations were seen more clearly and in some instances, helically coiled structures were found with a pitch comparable in dimensions to the peripheral lobulations of the particles in which the helices were not clearly visible (Figs. 5, 6). In some particles, a system of at least two helices could be seen distinctly and the constituent strands of each helix were themselves helically coiled (Fig. 7). Occasional particles were found whose widths were approximately $\frac{1}{4}$–$\frac{1}{3}$ that of the others. One is shown in Fig. 8 where the fragment is seen to contain a fairly regular helix whose gyres can in places be seen to be double i. e., to consist of two strands

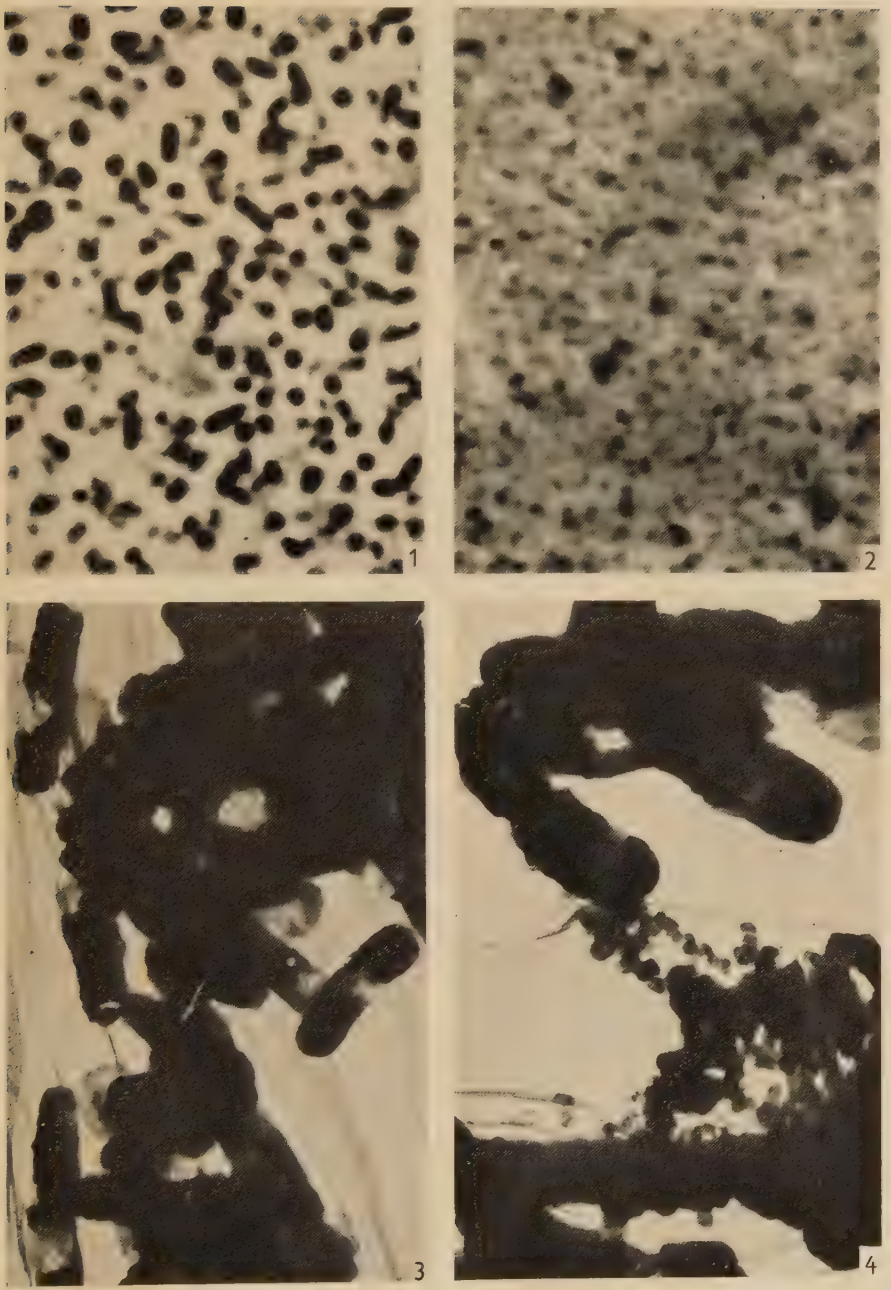


Fig. 1. Large particle fraction. Phase contrast. 3,400 ×.
Fig. 2. Small particle fraction. Phase contrast. 3,400 ×.
Fig. 3. Acetic acid-treated bacteria. Electron microscope. No shadowing. 6,000 ×.
Fig. 4. Coiled structure adjacent to disrupted cell. Acetic acid-treated bacteria. Electron microscope. 11,000 ×.

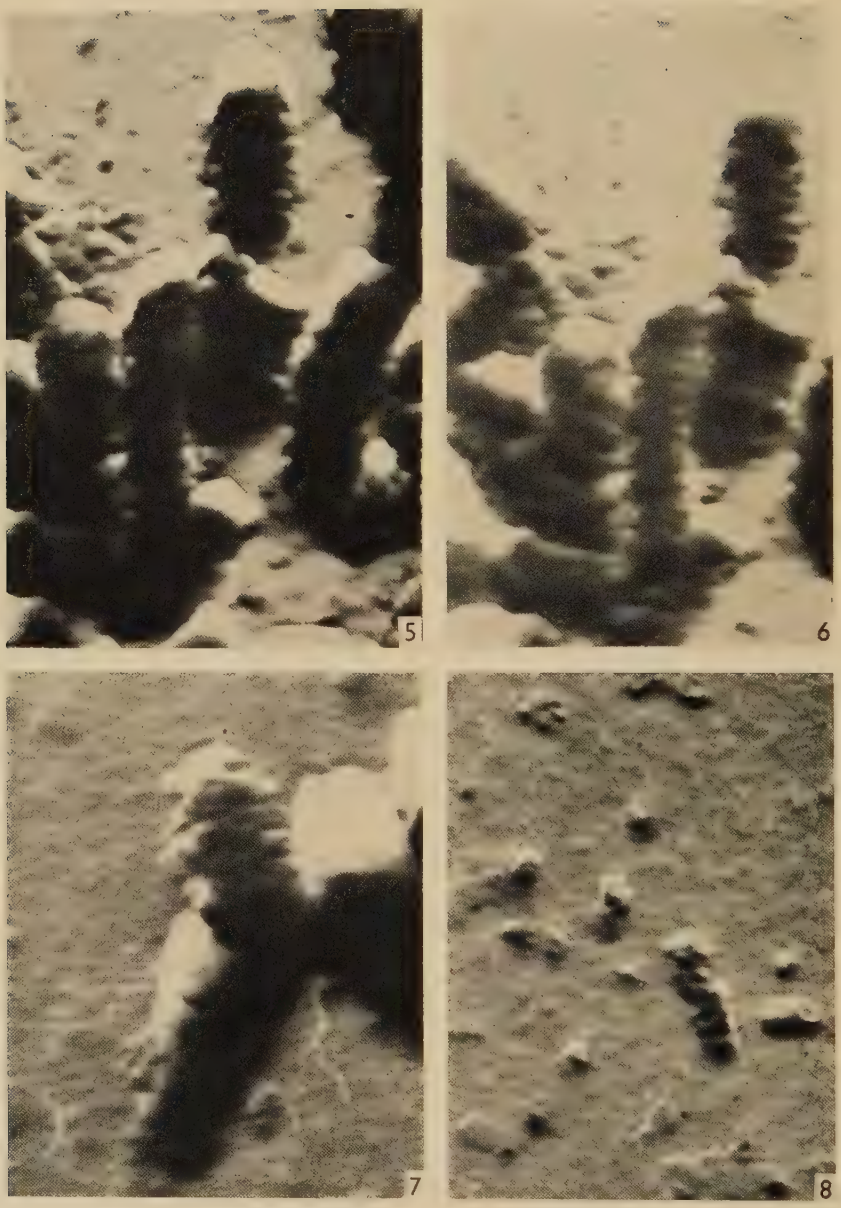


Fig. 5. Large particle fraction. Electron microscope. Chromium shadowing. 17,500×. Printed dark. Note peripheral lobulations.
Fig. 6. The same printed light to show coils in relation to lobulations.
Fig. 7. Particle showing helices whose constituent coils are themselves helically coiled. Electron microscope. Chromium shadowing. 31,500×.
Fig. 8. Fragment whose width is $\frac{1}{3}$–$\frac{1}{2}$ that of the average large particle. Note the two strands in each coil, and the helical coiling of each of the constituent strands (minor coiling). Electron microscope. Chromium shadowing. 17,500×.

rather than one. Where the individual strands are distinct, they can be seen themselves to be helices with a pitch approximately ½ that of the larger helix.

The average dimensions of the whole bacteria and of the particles as obtained from the electron micrographs are given in the following table:

Dimensions in μ

	length	width
Whole bacteria	2.91 ± 0.032	1.03 ± 0.012
Large particles	0.96 ± 0.002	0.63 ± 0.002
Small particles	0.28 ± 0.016	0.23 ± 0.020

The deviations given are standard deviations of the mean.

DISCUSSION

The average size of the large particles was approximately the same as that of the Feulgen-positive bodies of the intact bacteria and of those fragments obtained by sonic treatment which were found embedded in amorphous material. The size is also about the same as that of the Giemsa-staining bodies described by Hillier *et al.* (2). The lower base content per unit dry weight of the large particle fraction would at first sight appear to contradict the implication from the analogy in size, that there was a concentration of nuclear material in this fraction, for it is commonly assumed that there is a greater concentration of nucleic acid in nuclei than in cytoplasm. However the results obtained by Mudd and Smith (8) show very clearly that just the reverse is true in E. coli. They found that in the electron beam the nucleus was less dense than the bacterial cytoplasm, but after extraction of the ribonucleic acid, the density of the nucleus is then greater than that of the residual cytoplasm. The expectation from their observations is that on concentration of the nuclear material, there will be a lower nitrogenous base content in the concentrate than in the whole bacteria. The analytical results obtained here fulfill this expectation. It is also expected that there will be an increase in the relative amount of thymine, which also was found.

Where the helically coiled structure can be seen most clearly (Fig. 8), its diameter is found to be approximately 0.30 μ. In Fig. 6, the inner margins of the coils are not clearly seen but it is evident from the micrograph that there are two helical systems in these particles. Thus assuming that the diameter of each helix is the same, its dimension may be obtained from the width of the particles, and is found to be 0.32 μ, in agreement with the value

obtained above. Among the chromatinic bodies described by Hillier, Mudd, and Smith (2), are some which are clearly bipartite or bifurcate. Measurements from their illustrations give a mean value of 0.32 μ for the width of a single limb of these bodies. If the limbs of the bifurcate or bipartite bodies are considered to be chromosomes as has been previously assumed (9), then the above calculations suggest that the helices may be chromonemata. There is another important point of analogy in the clearly bipartite nature of the helix, since the chromonemata of higher organisms are also bipartite (4). In the higher plants, each component helix (the half-chromatid of the mitotic anaphase) is itself helically coiled (5). The electron micrographs of some of the E. coli particles show that each constituent "thread" of the large helix is itself helically coiled.

In addition to the morphological analogies indicated, the purine and pyrimidine analyses show that the particulate fraction containing these structures is nuclear material. Thus on the basis of their staining reactions, their dimensions, their morphology, and their nitrogenous base content, the bodies here described may be identified as the chromosomes of E. coli.

SUMMARY

1. Bacteria have been fragmented with sonic vibrations and the fragments separated into two fractions one containing the larger, the other the smaller particles.

2. Because of an obscuring black pigment, the large particles cannot be stained, but where they are embedded in a matrix of unknown origin and in the intact bacterium, they give a positive Feulgen reaction.

3. The large particle fraction has a lower adenine, guanine, and uracil content, and a higher thymine content, a result which is expected from previous investigations, if the nuclear material of the cells is concentrated in this fraction.

4. The average particle size in this fraction is approximately the same as that of the Feulgen- and Geimsa-staining bodies of E. coli which have been called nuclei.

5. Electron micrographs of the large particles show that they contain helical structures exactly analogous in number and morphology to the chromonemata of higher plants.

Supported by a grant from the Division of Research Grants and Fellowships of the U.S. Public Health Service.

REFERENCES

1. Breed, G. W., Breed-Bergey, E. D., and Swierenga, A. A., *Giorn. batteriol. immunol.*, **16**, 1 (1936).
2. Hillier, J., Mudd, S., and Smith, A. G., *J. Bact.*, **57**, 319 (1949).
3. Hutner, S. H., Provasoli, L., Schatz, A., and Haskins, C. P., *Proc. Am. Phil. Soc.* **2**, **94**, 152 (1950).
4. Kaufman, B. P., *Botan. Rev.*, **14**, 57 (1948).
5. Marshak, A., *J. Heredity*, **27**, 459 (1936).
6. Marshak, A., and Vogel, H. J., *Federation Proc.*, **9**, 85 (1950).
7. —— *J. Biol. Chem.* (1951). (In press.)
8. Mudd, S., and Smith, A. G., *J. Bact.*, **59**, 561 (1950).
9. Robinow, C. F., *Proc. Roy. Soc. London*, **130** B, 299 (1942).

ON THE CYTOPLASMIC CONSTITUENTS OF NORMAL TISSUE MAST CELLS[1]

BENGT SYLVÉN

Cancer Research Division of Radiumhemmet, Caroline Hospital and Caroline Institute, Stockholm, Sweden

Received November 26, 1950

The investigations to be reviewed were originally instigated by the observation of alcohol-resistant metachromatic material, provisionally interpreted as ester sulphate polysaccharides, in a large variety of growing mammalian tissues (7, 8, 16, 17, 18). Several years ago it was felt that such qualitative data had to be complemented by parallel chemical studies. From a cytochemical point of view, however, previously used extraction and separation methods were considered rough and inadequate. Our aim was to obtain the acid polysaccharides under question in a more native state and for this reason protein denaturation had to be avoided. For some time considerable attention was devoted to the extraction and study of polysaccharide material from the granulation tissue of healing wounds. We obtained mixtures of different protein-bound polysaccharides such as heparin, chondroitin sulphate and probably also hyaluronic acid (unpublished results), which at that time could neither be separated nor analysed. This necessitated the development of other methods for a more refined study of tissue polysaccharides.

First of all it seemed necessary to obtain more knowledge of the native heparin-bearing cytoplasmic constituents of normal tissue mast cells. Previously used extraction methods for heparin (1, 20) cause protein denaturation which leads to the artificial production of very firm linkages between heparin and proteins. For these and other reasons not stated in detail more attention had to be paid to the initial steps of the extraction procedures. A number of salt extraction methods were tried (13; partly unpublished), and finally we took up the method of Claude (2, 3) for fractionation of cytoplasmic constituents.

Preliminary Cytological Data. According to the conceptions advanced by Holmgren and Wilander (9) and Jorpes (11), the metachromatic heparin

[1] Paper presented before the VIIIth International Congress of Experimental Cytology in New Haven, September 4–8, 1950.

material was believed to be located in the large mast granules. If, however, living mast cells were treated by freeze-vacuum dehydration (Altmann-Gersh) and the sections stained with Azure A, Pinacyanol or Haematoxylin-Eosin and then seen under the light microscope we observed an amorphous intergranular material presenting a very pronounced metachromasia and eosinophilia (12), contrasting with the basophilia of the large mast granules. The existence of this amorphous material has been further demonstrated by phase-contrast microscopy of living rat mast cells (19). Additional data (unpublished) on the cytoplasmic structure of mast cells subjected to carcinogenic hydrocarbons and mastocytomas in dogs pointed in the same direction. Thus, we got reason to doubt the previous suggestion (9) as to the topic localization of the cytoplasmic metachromatic material. This led to a working hypothesis more in keeping with modern concepts of cytoplasmic structure (4), suggesting that the metachromatic material might be located in the intergranular cytoplasm as part of a microsome material. The unknown chemical composition of the large basophilic mast granules is subject to further research.

Differential Centrifugation. Fresh ox liver capsules with a mast cell content of about 40 per cent of the whole cell number were used as raw material. All metachromatic substance present in this material was located in the mast cell cytoplasm. The ground capsules were subjected to short time extraction and subsequent differential centrifugation of the extract in isotonic phosphate and other buffers at a uniform pH of 7.0–7.4 at + 4° C. For the diagram of flow and other details the readers are referred to previous report (12). Chemical analysis of different fractions showed that 80–85 per cent of the extracted metachromatic material was recovered as a non-sedimentable solute in the final supernate (S_3). The large mast granules had a higher weight and were easily sedimentable at low speed centrifugation. Their surface was coated by small amounts of metachromatic material which could be washed off. The microsome fractions sedimented at 40,000–60,000 × g contained about 10 per cent of the extracted amount of heparin. We did not attempt to get a high yield of heparin available in the raw material, since this would naturally entail the simultaneous extraction of other cell material. The present results are consequently based on the easily water-soluble heparin fractions obtained after initial extraction during 6–7 minutes.

The results indicate that the extracted cytoplasmic components had been segregated into (a) A large granules fraction, (b) A microsomes fraction, and (c) A final supernate (S_3). The latter material is provisionally regarded as dissolved from the microsomes fraction and its special composition may explain why this fraction is more water-soluble and non-sedimentable in contrast to the residual microsome material. Electron microscopical data show that the metachromatic and heparin-bearing material in S_3, with an

elementary diameter below 100 Å, was the only particulate matter present. Other data not presented in detail indicate that this particulate matter aggregates and is influenced by pH shifts in a similar manner as other microsome material (2, 3, 4).

The results so far obtained include converging cytological, chemical and bio-assay data. In view of possible objections against the differential centrifugation method as such, I want to emphasize that we applied this method to a more narrow and special problem than was done by Claude, not relating to microsome material on a whole. The metachromatic staining reaction provided good facilities for the identification of our cell material. Apart from this the chemical analyses and biological clotting assays of heparin corroborated our findings to an extent which renders conclusive interpretations possible. A tentative reconstruction of the submicroscopic structure of mast cell cytoplasm was consequently presented (12).

Composition of the Native Heparin Complex. Further investigations to be reported elsewhere (15) deal with analysis of the native heparin complex contained in the final supernate S_3. The results indicate that the particulate matter previously mentioned contains heparin associated with a lipoprotein material.

Isolated heparin has an average molecular weight of about 16,700 (6), but the more native heparin complex has a molecular weight of about 100,000. The lipoprotein part of this complex could be salted out by 65–80 per cent saturation with phosphate, leaving the polysaccharide residue in solution. The *lipid part* contained lecithin (55 per cent), cholesterol (8 per cent), and a residue of neutral fats (37 per cent). The *protein part* was a polypeptide with a molecular weight of about 8,000 and a pattern of only seven amino acids, namely cysteine (cystine), threonine, tyrosine, glycine, leucine, tryptophane, and arginine. The *carbohydrate residue* in solution was inhomogeneous and could be segregated into two or three subfractions (10, 14).

The whole material contained in S_3 could by means of electrophoresis at pH 7 be segregated into 3 different fractions with the mobilities of $u_6 = 5.9$, $u_4 = 3.6$ and $u_3 = 2.7 \times 10^{-5}$ sq. cm per volt per second. In this native supernate no component was found with the mobility of isolated heparin. The first mentioned component u_6 was the only one exerting antithrombic and metachromatic activities. Further studies including salting out experiments, quantitative electrophoretic separation, and detailed chemical and antithrombic assays clearly indicated that fraction u_6 (sedimentation constant of about 4.4) was made up of heparin + a polypeptide part + a lipid component. The polypeptide contained six amino acids, the same as mentioned

above except arginine, and presented physico-chemical and biological characteristics identical with those of the previously called heparin complement of serum. This lipoprotein part loosely associated with the heparin moiety in the native state, was thus identified by means of its biological activity as a true heparin complement obtained from tissue mast cells. Isolation and analysis of the heparin complement (heparin co-factor) of serum, which is necessary for the final identification, has however not yet been performed.

It may further be mentioned that this native heparin complex has also been found to exert a similar high anti-hyaluronidase activity (5) as isolated heparin.

This heparin complex (u_6), including the identified complement-active lipoprotein, is most likely synthesized and produced in the mast cell cytoplasm. The present results illustrate the value of close co-operation between cytological and physico-chemical lines of research.

ACKNOWLEDGEMENTS

The efforts and skilfulness of my co-workers Olle Snellman, Ph.D., and Miss Christina Julén, B.A., are gratefully appreciated. I want to express my sincere gratitude to Prof. The Svedberg, and to Prof. Arne Tiselius, University of Uppsala, who have generously placed their institutes and research facilities at our disposal. The costs have largely been defrayed by grants from private donors, and further from the Swedish Medical Association, and the Swedish Cancer Research Funds.

REFERENCES

1. Charles, A. F., and Scott, D. A., *J. Biol. Chem.*, **102**, 425 (1933).
2. Claude, A., *J. Exptl. Med.*, **80**, 19 (1944).
3. —— *J. Exptl. Med.*, **84**, 51 and 61 (1946).
4. —— *Advances in Protein Chemistry*, **5**, 423 (1949).
5. Glick, D., and Sylvén, B., *Science* **113**, 388 (1951). (In press.)
6. Grönwall, A., Ingelman, B., and Mosimann, H., *Upsala Läkareför. Förh.*, **50**, 397 (1945).
7. Holmgren, H., *Anat. Anz.*, **88**, 246 (1939).
8. —— *Z. mikroskop. anat. Forsch.*, **47**, 489 (1940).
9. Holmgren, H., and Wilander, O., *Z. mikroskop. anat. Forsch.*, **42**, 242 (1937).
10. Jensen, R., Snellman, O., and Sylvén, B., *J. Biol. Chem.*, **174**, 265 (1948).
11. Jorpes, J. E., in Heparin in the Treatment of Thrombosis. London, Oxford University Press, 1946.
12. Julén, C., Snellman, O., and Sylvén, B., *Acta Physiol. Scand.*, **19**, 289 (1950).
13. Snellman, O., Jensen, R., and Sylvén, B., *Nature*, **161**, 639 (1948).
14. —— *Acta. Chem. Scand.*, **3**, 589 (1949).
15. Snellman, O., Sylvén, B. and Julén, C., *Biochim. et Biophys. Acta*, **7** (1951). (In press.)
16. Sylvén, B., *Klin. Wochschr.*, **17**, 1545 (1938).
17. —— *Acta Chir. Scand.*, Suppl. 66, **86** (1941).
18. —— *Acta Radiol., Suppl.* **59**, 1945.
19. —— *Exp. Cell Res.*, **1**, 492 (1950).
20. Wilander, O., *Skand. Arch. Physiol.*, Suppl. 15, **81** (1938).

RYANODINE AND CONTRACTILE PHENOMENA IN THE MITOTIC SPINDLE[1]

IVOR CORNMAN

George Washington University Cancer Clinic and the Department of Anatomy, School of Medicine, Washington[2], D. C.

Received November 29, 1950

There is good evidence for the existence of mitotic traction fibers (2) and for their contraction (4, 9), but the experimental attack on the nature of the contraction which pulls the chromosome has been retarded by the fact that the most efficient mitotic poisons completely destroy the spindle. This is being remedied by studies such as those presented at this meeting by Hughes, wherein the effects of a number of substances on all the phases of mitosis are analysed.

Another substance which conceivably could shed some light on contractile processes is the alkaloid, ryanodine, which appears to inhibit the contraction process itself in striated muscle. Edwards, *et al* suggest that the point of interference by ryanodine is at the ATP-ADP-actomyosin cycle (3). There is evidence that adenosine triphosphate is involved in cell division. The work of Runnström clearly reveals the participation of contractile forces which operate at fertilization and which are aided by ATP. Moreover, cleavage can be improved in eggs which cleave poorly as a result of natural conditions (10) or where inhibition has been imposed experimentally with hypertonicity or dinitrophenol (11). Particularly pertient to the work reported here is Runnström's observation that in subnormal eggs the separation of the nuclei is incomplete, but that normal chromosome migration ensues when ATP is added (11). Landschütz has demonstrated that ATP-ase activity is quantitatively related to mitotic activity in some tumor tissues (5), and further that ATP is antagonistic to colchicine (6). Lettré has also found that ATP will alleviate colchicine damage to the spindle (7). These findings give encourage-

[1] Paper presented before the VIIth International Congress of Experimental Cytology in New Haven, September 4–8, 1950.

[2] This work was aided by a grant from the Washington Heart Association and by an American Cancer Society institutional grant to the George Washington University Cancer Clinic.

ment to the idea of challenging the contractile spindle fiber with ryanodine, an encouragement furthered by Brachet's speculations as to similarities in the contraction of spindle and muscle fibers (1).

MOUSE HEART IN TISSUE CULTURE

Method

In order to test the effects of ryanodine on cell division at the concentrations which inhibit contraction, cultures were prepared from hearts of newborn Strain C mice. Pulsating fragments were cultured for two days in nutrient solution (4 parts Gey's saline + 1 part human placental serum + 1 part 20 per cent embryo extract). This was replaced with 0.5 ml of fresh nutrient containing one-half or one mg of ryanodine. The progressive weakening of the pulsations was recorded and cultures were fixed at intervals for subsequent counts of the mitoses. The cultures were fixed in Zenker's and stained by the Feulgen technique. Satisfactory counts can be made of roller tube cultures using thin-walled tubes and a 45 × oil-immersion objective with a long working distance.

Results

In preliminary experiments using gradually increased concentrations of ryanodine it was found that 1 to 2 mg/ml was required to stop pulsation of the fragments. The blocked fragments did not respond to epinephrine.

Following the addition of fresh nutrient the mitotic activity of normal cultures increases, as the control shows in Experiment I, Table I. In the presence of a threshold dose of ryanodine (1 mg/ml) the increase was prevented (Experiment II, Table I) and sooner or later the mitotic count declined (Experiments I and II). At 2 mg/ml the frequency of mitoses decreased more sharply.

At both concentrations there was an accumulation of blocked mitoses (B). This category comprised cells containing thick, disoriented chromosomes. Most of the blocked cells were metaphases (Fig. 1, left) although a few appeared to derive from anaphases (Fig. 1, right) and telophases. While some figures in the control cultures could be classified as aberrant (and every variation from the typically normal configuration in the controls was counted as "blocked"), the percentage ranged from one to seven, as against 17 per cent to 58 per cent blocked by the low dose of ryanodine and 37 per cent to 100 per cent blocked at 2 mg/ml. Anaphases were not increased by ryanodine treatment, although any slight increase would be obscured by the practise of counting any borderline metaphase or telophase as anaphase in the control to weight against the experimental trend.

TABLE I

Mitotic suppression and interruption in heart fibroblasts by ryanodine.

Experiment I.

	Control			1 mg/ml.		2 mg/ml.	
	0 hrs.	2 hrs. 20′	4 hrs.	2 hrs. 20′	4 hrs.	1 hr.	2 hrs. 20′
P	13	27	107	1	7	3	0
M	19	25	81	3	5	8	2
B	4	2	4	5	6	14	11
A	5	9	94	4	9	5	1
T	12	10	96	9	8	8	1
Total	53	73	382	22	35	38	15
Per cent B	8	3	1	23	17	37	73

Experiment II

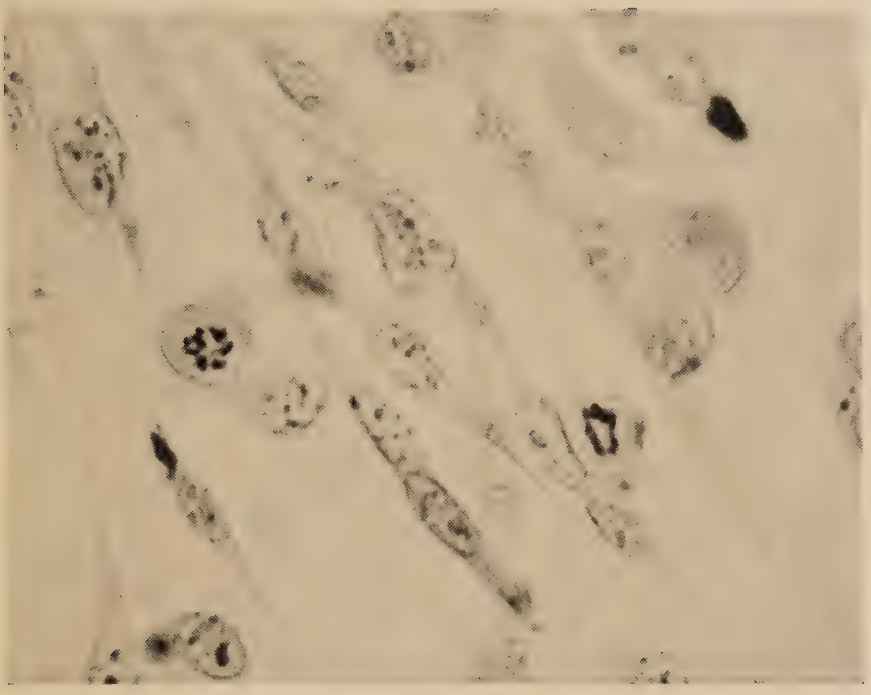
	Control	1 mg/ml.			2 mg/ml.	
	7 hrs.	2 hrs. 20′	4 hrs. 15′	7 hrs.	2 hrs. 20′	4 hrs. 15′
P	166	11	45	4	3	0
M	102	10	38	3	1	0
B	18	24	36	14	20	6
A	104	5	17	3	0	0
T	34	3	11	0	0	0
Total	424	53	147	24	24	6
Per cent B	4	45	25	58	83	100

Each column represents total counts of 10 cultures in Expt. I, and 20 cultures in Expt. II. P = Late prophase (definite chromosomes within the nucleus, or unoriented in the cytoplasm). M = Metaphase (equatorial plate). B = Blocked (thick disoriented chromosomes or pycnotic clumps). A = Anaphase (2 well-defined chromosome clusters). T = Telophase (distinct furrow).

DIVIDING SAND-DOLLAR EGGS

Method

In order to examine more closely the effect of ryanodine on chromosome movement, the course of mitosis was followed in eggs of *Echinarachnius parma*, where the living asters, spindle, and chromosomes can be observed. At various times after fertilization the eggs were exposed to ryanodine concentrations ranging from 6 to 16 mg/ml. As cleavage progressed, counts were made and plotted to determine the time at which half the eggs had divided and the total percentage of divided eggs.



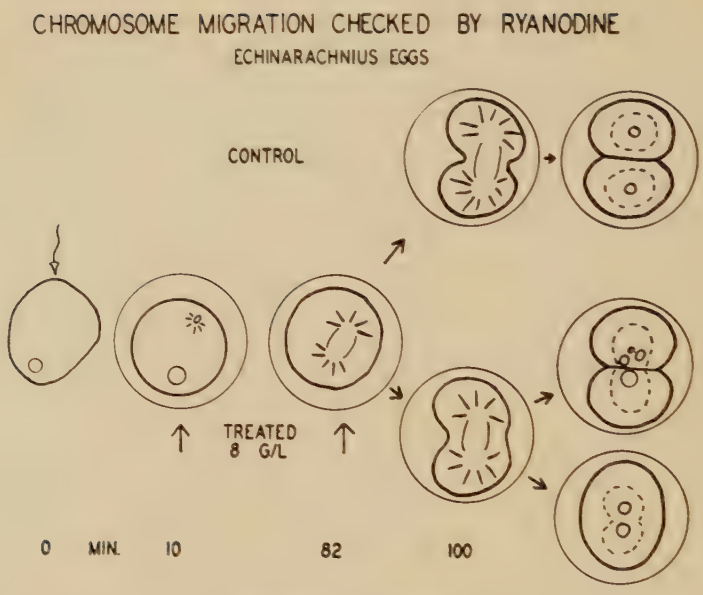
Explanation of Figure 1.

Thick, disoriented chromosomes of a metaphase (left) and anaphase (right) in mouse heart fibroblasts following 2 hrs. and 20 min. exposure to ryanodine, 2 mg/ml.

Results

The effects of early exposure to different concentrations are shown in Experiment I, Table II. The percentage of eggs dividing was sharply decreased by small increases in concentration in this range. The time at which half the eggs had completed division was also increased, although this cannot be measured for 8 mg/ml, inasmuch as fewer than half the eggs divided. These eggs reached their maximum of 14 per cent cleavage 119 minutes after fertilization. Total cleavage was less affected and retardation was accentuated when the eggs were exposed later in the mitotic cycle. In Experiment II (Table II), the average retardation was 20 minutes when exposure began at 77 $\frac{1}{2}$ minutes after fertilization, whereas 60 per cent of the eggs divided.

Some eggs which furrowed deeply and were to all appearances 2-celled eventually lost their furrows. This was more striking with late dosage. In



Explanation of Figure 2.

The normal course of cleavage as observed in living sand-dollar eggs is shown at the left and the upper right. If ryanodine is added when the earliest eggs are beginning to cleave (82 min.) division of the eggs is retarded. Some eggs divide, some form furrows which regress and some do not attempt cleavage. The resulting nuclei or karyomeres and their surrounding lakes of achromatic material are separated by only a fraction of the distance traversed in normal eggs. On much longer exposure (beginning at 10 min.) the effects are qualitatively the same, but somewhat more severe (cf. Table II).

TABLE II

Cleavage of Echinarachnius eggs in ryanodine.

Experiment I. Ryanodine 10 1/4 min. after fertilization				Experiment II. Ryanodine conc. 8 mg ml.			
Dose	Minutes to 44 per cent cleavage	Maximum cleavage in per cent	Final cleavage in per cent	Dosage begun	Minutes to 46 per cent cleavage	Maximum cleavage in per cent	Final cleavage in per cent
Control.....	99	88	88	Control	102	92	92
6 mg/ml.	112	52	42	77 1/2 min. ..	122	60	25
8 mg/ml.	(119)	14	8	81 3/4 min. ..	116	76	56

In Experiment I the duration of exposure is constant and the concentration is varied. In Experiment II the concentration is constant and the time of exposure is varied. The beginning of exposure and the halfway point of cleavage is measured from the moment of fertilization. In Experiment I, dose 8 mg ml. the time of maximal cleavage is given inasmuch as less than half the eggs divided. In the treated eggs furrows regressed after nearly dividing the eggs. The amount of regression is indicated by the difference between *Final Cleavage* and *Maximum Cleavage.*

Experiment II (Table II) an apparent cleavage of 60 per cent regressed to 25 per cent. Simultaneously some 2-cell stages prepared for a second division. About half the late-exposed eggs divided again, and some reached the 8-cell stage.

The closeness of the nuclei in the binucleate eggs resulting from furrow regression is evidence of poor chromosome separation. Even in eggs which divided completely, the new nucleus or the cluster of karyomeres which sometimes appeared in place of the nucleus, were close to the new surface. Regression of the furrow and inhibition of chromosome migration are diagrammed in Figure 2.

At 16 mg/ml a small amphiastral figure appeared and the nucleus dissolved, but the chromosomes remained in the center of the egg, eventually forming one nucleus or several karyomeres.

DISCUSSION

The concentration of ryanodine required to block the spontaneous contraction of heart muscle is about a hundred times that which Pogogeff and Murray found would block skeletal muscle in cultures (8). The amount of ryanodine which stopped the pulsations also blocked the mitoses, but it simultaneously prevented inception of new mitoses.

In the introduction strong circumstantial evidence was presented for the similarity of muscle fibril and traction fiber contraction, pointing to a key role of ATP in both. The experiments with ryanodine add to the evidence. Blocking of mitoses in the cultured heart cells is consistent with an interference with chromosome migration, but could just as well represent an indiscriminate destruction of the spindle.

The evidence supplied by the dividing eggs is somewhat more specific, showing that chromosome separation is indeed slowed and incomplete at concentrations which permit the completion of several divisions. In Table II the effect of 6 mg/ml beginning an hour and a half before cleavage is about equivalent to exposure to 8 mg/ml during mitosis. It appears that the premitotic processes are relatively little affected, and that ryanodine is not a generally toxic substance, but is specifically interfering with mitosis. This still does not prove that ryanodine inhibits contraction of the spindle fiber, although all the results are consistent with an effect of that type. Polarized light and motion picture studies should give a more definitive answer, and it is to be hoped that investigators with motion picture technique at their command will find the problem worth attention.

SUMMARY

Spontaneous contraction of mouse heart muscle in tissue culture is stopped by ryanodine at 1–2 mg/ml. These concentrations also block mitosis. In the dividing *Echinarachnius* egg, anaphase movement of chromosomes is retarded and incomplete in ryanodine at 6–8 mg/ml, although some of the eggs complete 2 or 3 cleavages. Increasing the length of exposure prior to cleavage does not alter the character of response and only slightly increases the severity. These phenomena are consistent with an inhibition of traction fiber contraction by ryanodine, but are insufficient to demonstrate the existence of such a contractile phenomenon.

REFERENCES

1. Brachet, J., Embryologie Chimique, Masson & Cie, Paris, 1947, p. 200.
2. Cornman, I., *Am. Nat.* **78**, 410 (1944).
3. Edwards, G. A., Weiant, E. A., Slocombe, A. G., and Roeder, K. D., *Science*, **108**, 330 (1948).
4. Hughes, A. F., and Swann, M. M., *J. Exptl. Biol.*, **25**, 45 (1948).
5. Landschütz C., *Experientia*, **6**, 232 (1950).
6. —— Personal communication.
7. Lettré, H., *Ergeb. Physiol. Biol. Chem. Exptl. Pharmakol.*, **46**, 379 (1950).
8. Pogogeff, I. A., and Murray, M. R., *Anat. Rec.*, **103**, 495 (1949).
9. Ris, H., *Biol. Bull.*, **96**, 90 (1949).
10. Runnström, J., *Exp. Cell Res.*, **1**, 497 (1950).
11. Runnström, J., and Kriszat, A., Personal communication.

ISOLATED CHROMOSOMES

HANS RIS and A. E. MIRSKY

Department of Zoology, University of Wisconsin, Madison, Wisc., and The Rockefeller Institute for Medical Research, New York

Received December 29, 1950

In a preceding paper (3) as well as in an earlier note (2) Lamb has claimed that the nucleoprotein threads isolated from vertebrate tissues were random fragments of drawn out nuclei and not isolated chromosomes. We would like to take this opportunity to summarize the evidence, partly given in a previous paper (7), which we think shows that the threads are chromosomes liberated from interphase nuclei.

Let us look at the isolated threads and compare them with the picture we have of chromosomes in the interphase nucleus.

1) Isolated threads show longitudinal differentiation into thin irregularly twisted regions and thicker, darkly staining regions. Similar structures are already visible in intact nuclei, Fig. 8.

2) Isolated threads are often clearly double (7), Fig. 1. Sometimes the doubleness is visible only in part of the structure where the two chromatids are widely separated. Such threads are often Y-shaped (7), Fig. 4. We cannot agree therefore with Lamb, that the doubleness is simply an optical illusion. Isolated threads are as clearly double as chromosomes in anaphase of mitosis, a fact now accepted by all cytologists. The double structure of isolated threads was also observed by Claude and Potter (1).

3) The thicker regions on the threads contain tightly spiralled chromonemata which are easily revealed with uncoiling agents (KCN, etc., (7)), Figs. 7 and 8.

4) The threads have individuality expressed in specific size and shape of the thin and thick regions. Chromosomes of the same type can be repeatedly recognized, even in different tissues of the same species, Figs. 1, 2, 3, 4. Beef chromosomes in mitosis all have the kinetochore near one end (6).

Isolated threads from beef tissues commonly have thin regions at one or both ends which are often clearly double. One of these probably corresponds to the short arm of the chromosome. In horse (4) and sheep (5) metacentric chromosomes as well are found during mitosis. Threads isolated from tissues of these animals often show a clear gap between two thick regions, probably corresponding to the kinetochor. Such constrictions were never observed in threads from beef tissues. These observations of individuality of isolated threads disagree with the contention of Lamb that the threads are of arbitrary length and shape and are strong evidence in favor of the chromosome nature of isolated threads.

5) The individuality of the isolated threads is most beautifully shown by the nucleolus organizing chromosome. The nucleolus, as is well known, is attached to a certain region of a specific chromosome. Isolated threads with attached nucleoli were repeatedly found, Figs. 5, 7. They have an identical structure in preparations from either beef liver or pancreas. The nucleolus is attached to a secondary constriction near one end of the chromosome which carries a small trabant. On the other end a thin short thread is present (short arm?). The nucleolus here acts as a tag for a specific chromosome (one of the nucleolus organizing chromosomes) which can be found repeatedly among isolated threads. It is difficult to imagine how random fragmentation of stretched nuclei could produce this structure.

Of course we do not mean to say that all threads are individual chromosomes. No doubt sticking together, distortion and fragmentation do occur. We have observed structures that are too large to be individual chromosomes. Some of these large bodies may be stretched nuclei. The important point is that a high percentage of the threads show all the characteristics expected of interphase chromosomes, and, above all, individuality of structure that could not be produced by random fragmentation.

It has sometimes been suggested that chromosomal structures in the interphase nucleus as well as the isolated threads might be artefacts since no structures are visible in the uninjured living nucleus. We have discussed this question in a previous paper (9) where it was shown that the homogeneity of the interphase nucleus is due to the fact that the chromosomes exist in an extended state and fill the entire nucleus. This absence of visible structure, therefore, does not contradict the individual persistence of the chromosomes in the non-dividing nucleus.

Finally we would like to make some remarks concerning specific points in Lamb's paper (3). 1. Distorted nuclei: We have observed small numbers of distorted nuclei in our preparations. We cannot agree, however, that all

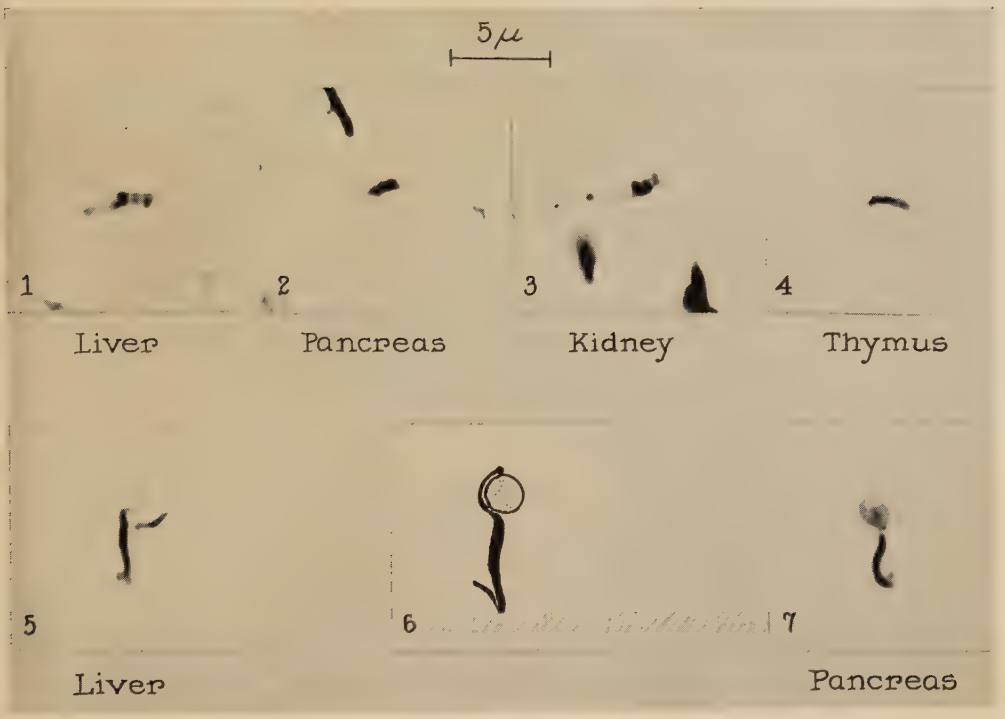
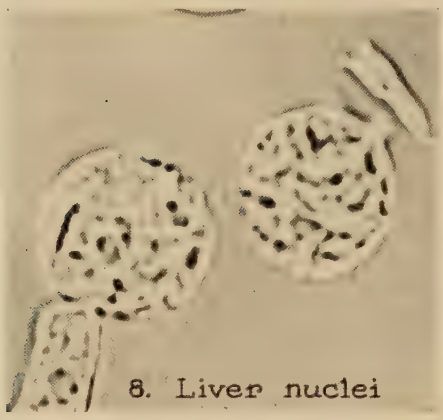


Plate I.

The isolated chromosomes on Plate I were all fixed and stained in aceto-orcein. They were photographed with a 2 mm Zeiss apochromat, 15 × ocular—magnification 1,200 ×. Since they were enlarged 2 × photographically, the final magnification is 2,400 ×. Figure 6 is a schematic drawing of the nucleolar chromosomes. Figure 8 is a photograph of beef liver citric acid nuclei, unstained, magnification 2,400 ×.

The figures of isolated chromosomes on this plate are taken from a paper by Mirsky and Ris which will soon appear in the Journal of General Physiology. They are reproduced here by permission of the editors of the Journal of General Physiology.

nuclei are distorted first and then fragmented. After short runs in the blender a large number of normal intact nuclei and some isolated threads are present. With continued treatment the intact nuclei decrease in number until only isolated threads are present. The isolated threads are also of the same size and shape after short or longer treatment in the blender. We found no evidence of fragmentation with prolonged treatment. 2. The isolated threads of our preparations are always entirely Feulgen positive. They certainly cannot be described as threads which have "Feulgen positive regions here



and there along their length," or which contain "none" such regions, as Lamb says. 3. A comparison with metaphase chromosomes is meaningless. It is evident that a chromosome will look different in interphase than in metaphase. One might also expect a greater visible individuality in longitudinal differentiation in the interphase than in the evenly and tightly coiled metaphase chromosomes. 4. Use of the electron microscope. The electron microscope may be useful in the future for a study of the detailed structure of chromosomes. So far, however, it has not yielded any definite information on the structure of mitotic chromosomes. It is at present, therefore, not an adequate tool for the recognition of chromosomal structures. 5. Are the structures described by Lamb comparable to our "isolated chromosomes"? Judging from his photographs his preparations look quite different from ours (7), Figs. 1-13. He might even be correct in concluding that in his preparations distortion and fragmentation of nuclei is common. Whether this is the case with preparations of other investigators has to be judged in each case from the type of structures produced. Our procedure has been published earlier (7) and recent modifications are to appear shortly in the Journal of General Physiology.

Recently Pfeiffer (8) isolated chromatin threads from cultures obtained from follicle epithelium of Drosophila spec. He describes the threads as having primary constrictions and often also secondary constrictions. With regard to the chromosome nature of isolated threads he comes to the following conclusions: "If we survey all the investigations as a whole we are inclined to consider the chromatin threads isolated by Mirsky as being true chromosomes of intermitotic nuclei."

SUMMARY

Nucleoprotein threads isolated from mammalian tissues are interphase chromosomes rather than random fragments of stretched nuclei. Structures similar to the isolated threads are visible in intact nuclei. The isolated threads are often clearly microscopically double, consist of more or less tightly coiled chromonemata and show individuality in longitudinal differentiation. A nucleolus organizing chromosome with trabant and secondary constriction carrying the nucleolus can be recognized and is of identical structure in preparations from beef liver and pancreas.

REFERENCES

1. Claude, A., and Potter, J. S., *J. Exp. Med.*, **77**, 345 (1943).
2. Lamb, W. G. P., *Nature*, **164**, 109 (1949).
3. —— *Exp. Cell Res.*, **1**, 571 (1950).
4. Makino, S., *Cytologia*, **13**, 26 (1943).
5. —— *Cytologia*, **13**, 39 (1943).
6. —— *Cytologia*, **13**, 247 (1944).
7. Mirsky, A. E., and Ris, H., *J. Gen. Physiol.*, **31**, 1 (1947).
8. Pfeiffer, H. H., *Experientia*, **6**, 334 (1950).
9. Ris, H., and Mirsky, A. E., *J. Gen. Physiol.*, **32**, 489 (1949).

STUDIES ON THE ULTRASTRUCTURE OF BONE

ARNE ENGSTRÖM and ROLF ZETTERSTRÖM

Department for Cell Research, Karolinska Institutet, and Department for Metabolic Research, the Wenner-Gren Institute, University of Stockholm, Sweden

Received January 25, 1951

Despite the great number of investigations concerning the nature of the inorganic constituents of bone and its ultrastructure, the problem of fine structure still remains obscure. For relevant references see Fankuchen (8), Brandenberger and Schinz (2), and Dallemagne (5). In general the x-ray diffraction techniques applied have been the Debye-Scherrer technique for obtaining diffractograms of powdered bone and the fiber diffractogram technique. Relatively wide pinholes have been used for the collimation of the x-rays.

The use of a micro-diffraction technique would make it possible to get diffraction patterns from localized areas of the bone such as single Haversian systems or pieces of primary and other secondary bone. The present investigation evaluates the possibility of detecting differences in the structure and orientation of the mineral salts in different parts of bone in normal and certain pathological conditions.

MATERIAL

Calcium phosphates of different composition and solubility were precipitated from salt solutions according to Kuyper (10). The composition of the solutions from which the compounds were formed is listed in Table I. Table II shows the chemical composition of the powders obtained.

Longitudinal and cross sections of the compacta of the long bones from different animals were prepared by grinding the bones under absolute ethanol. About 100 μ thick sections were used.

Almost complete decalcification of the sections was obtained after treatment with nitric acid as described by Amprino.[1]

Hyperparathyroidism was induced by injecting 2–3 months old dogs with heavy doses of parathormone (PTH) (7).

Rickets was produced in a two months old dog by giving a rachitogenic diet according to Mellanby (12) for five weeks. Severe rickets could be demonstrated by x-ray examination.

[1] Personal communication.

TABLE I

Sample Nr	Diffraction Nr	Time of Equilibration in Hours	Composition of the Solutions					pH
			Ca	P	Mg	Citrate	$NaH\ CO_3$	
			M	*M*	*M*	*M*	*M*	
1[1]	D 70. D 71	5	0.005	0.003			0.025	7.4
2	D 73	7	0.005	0.003			0.025	7.4
3	D 74	36	0.005	0.003			0.025	7.4
4	D 76	36	0.005	0.003	0.0037		0.025	7.4
5	D 75	36	0.005	0.003		0.00029	0.025	7.4
6	D 72	36	0.005	0.003	0.0037	0.00029	0.025	7.4

Composition of the solutions from which the precipitates are formed and time of equilibration.

In addition sufficient sodium chloride was present in all solutions to bring the ionic strength to 0.158. To prevent precipitation immediately upon addition of sodium bicarbonate, the solutions were presaturated with carbon dioxide gas. The solutions were placed in a constant temperature bath at 38° C and stirred continuously by bubbling through them a mixture of carbon dioxide and air. Gases were saturated with water vapor at 38° C before they were passed through the experimental solutions. The whole volume was 200 ml for each mixture.

TABLE II

Sample Nr	Diffraction Nr	Ca:P	CO_2	Mg	Citrate	Composition of the Supernatants after Precipitation[2]	
						Ca	P
			Per cent	Per cent	Per cent	mg/100 ml	mg/100 ml
1	D 70. D 71	1.50	8.5			2.73	1.58
2	D 73	1.47	4.5			4.59	2.17
3	D 74	1.53	6.2			2.22	1.25
4	D 76	1.33	6.2	3	1.1	4.72	1.76
5	D 75	1.54	6.7		1.2	3.37	2.07
6	D 72	1.34	4.9	3		5.03	2.09

The composition of the precipitates

Calcium and magnesium were determined in the Beckman flame spectrophotometer at the wave-lengths 422.7 and 285.2, respectively. Phosphate was determined according to Fiske and Subbarow (9), citrate with the pentabromaceton method (13) and carbon dioxide after treatment of the sample with hydrochloric acid and precipitation of CO_2 with barium hydroxide.

[1] In this sample the solution was not presaturated with carbon dioxide gas before addition of sodium bicarbonate.

[2] These values have been used as an index of the solubility of the different precipitates as, according to the work of Kuyper (11), there seems to be good relation between those values and the solubility.

TECHNIQUE

The x-ray microcamera described by Chesley (3) was used. A pinhole 34 μ in width was used as collimator. The distance between the film and the specimen was 9.85 mm. The camera was calibrated with rocksalt. Kodak No Screen x-ray film was used.

Philip's x-ray diffraction unit with a copper target x-ray tube was used as source of radiation. A Ni filter surpressed the Cu Kβ radiation. Cu Kα of the wave-length 1.54 Å was used.

Bone samples investigated were mounted over the slit by means of a movable holder. In that way the structure for analysis could be selected and checked under a microscope at about 50 times magnification.

The microcamera cannot be used for high precision measurements as all distances are small, and a small change in the distance between sample and film gives changes in the diameter of the diffraction rings and arcs. In a spacing of 2.5–3 Å the estimated error is ± 0.02 Å.

RESULTS

Table III gives the interplanar spacings of the different calcium phosphate compounds described in Table II. The strongest ring is the one corresponding to the spacing 2.76 Å. As this ring is relatively broad it occupies a "spacing distance" of 0.06–0.07 Å. There is a relatively diffuse ring not included in

TABLE III

Sample Nr	1		2	3	4	5	6
Diffraction Nr	D 70	D 71	D 73	D 74	D 76	D 75	D 72
Interplanar Spacing Å	1.70	[1]	[1]	1.70	1.69	1.70	1.71
	1.82	1.82	1.78	1.82	1.83	1.82	1.82
	1.93	1.93	1.96	1.93	1.94	1.93	1.93
	2.23	2.28	2.27	2.24	2.27	2.25	2.25
	2.73	2.76	2.77	2.77	2.76	2.76	2.82
	3.08	3.05	3.10	3.07	3.07	3.08	3.11
	3.43	3.39	3.42	3.40	3.40	3.42	3.44
	3.88	3.90	3.87	3.85	3.85	3.85	3.84

Interplanar spacings of the powders classified in Table II

Technique as described in the text.

[1] Too weak to be measured with accuracy. Some other weak lines eg. 2.61 and 2.06 Å have been omitted in this table as the spacings cannot be determined accurately. In case of sample 1 the reproducibility was checked by running several samples also with Mo Kα as radiation.

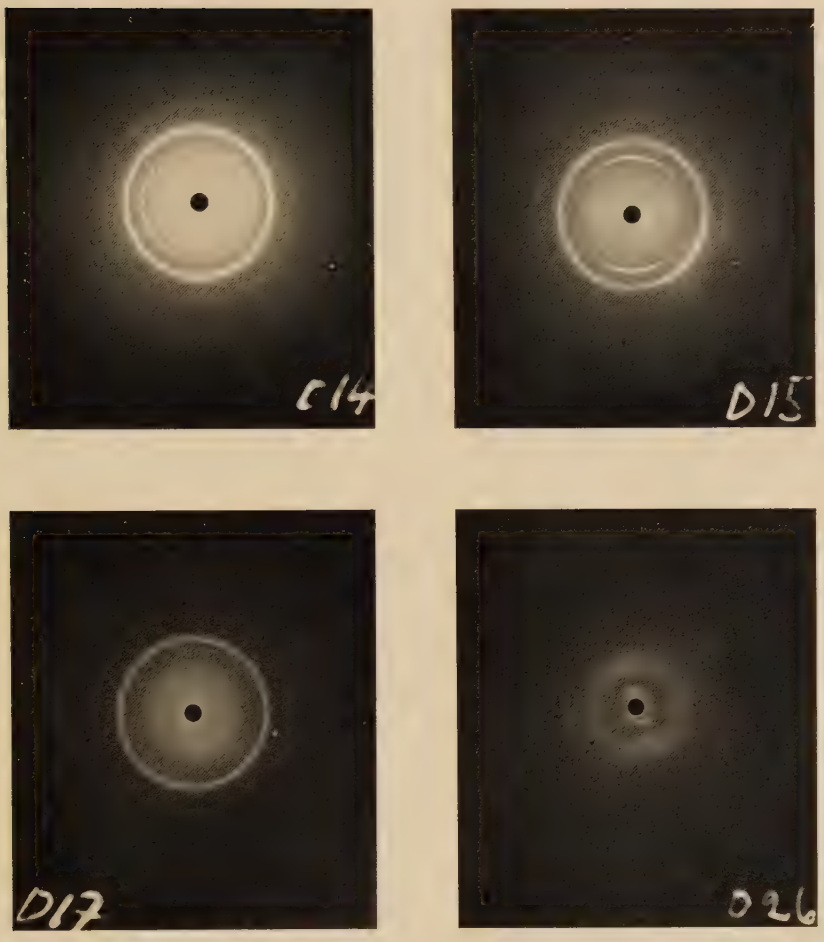


Fig. 1. Diffractograms of thin sections of bone.

Cu Kα-radiation filtered in Ni.
D 14. Human, newborn, secondary bone; longitudinal section.
D 15. Calcified tendon, hen.
D 17. Horse, metacarpus, cross section.
D 26. Horse, metacarpus, longitudinal section, decalcified.

Note the longitudinal orientation of the diffraction rings in D 14 and D 15. In D 26 the strong ring is oriented perpendicular to the collagen arcs near centrum. The diffuse ring is also oriented.

the table at about 5 5.5 Å in all samples. There seems to be no significant difference between the different compounds. The differences present are due to experimental errors.

Table IV gives the interplanar spacings for some bone structures. The

TABLE IV

Animal	Dog			Man				Cow	Hen
Bone	Femur Rachitis	Radius Normal	Radius Immobilized	Femur PTH	Newborn Primary Bone	New Osteon 45 yrs	Old Osteon 45 yrs	Metacarpus Secondary Bone	Ossified Tendon
Diffractogramm	D 78	D 9	D 8	D 63	D 13	D 10	D 11	D 21	D 16
	3.75	3.76	3.76	3.86	3.78	3.70	3.77	3.70	3.70
	3.38	3.39	3.35	3.42	3.36	3.34	3.38	3.32	3.35
	3.06	3.08	3.09	3.13	3.03	2.98	2.99	3.04	3.09
	2.74	2.74	2.72	2.77	2.75	2.71	2.74	2.72	2.71
Interplanar	2.65	2.61	2.58	2.59	2.59	2.60	2.62	2.60	2.59
Spacing Å	2.23	2.25	2.22	2.24	2.22	2.22	2.24	2.24	2.28
	2.02	2.05	2.03	2.05	2.03	2.02	2.04	2.01	2.06
	[1]	1.93	1.91	1.91	1.91	1.91	1.92	1.91	1.92
	1.82	1.82	1.82	1.85	1.82	1.80	1.82	1.81	1.80
	1.70	1.70	1.70	1.70	1.69	1.69	1.70	1.69	1.69

Interplanar spacings of different structures in bone

Diffractograms D 8, D 9, D 10, D 11, D 13, D 16 and D 21 are taken from Amprino and Engström (1), and D 63 from Engström and Engfeldt (7a). Technique as described in the text.

longitudinal sections showed strong orientation of several rings, *cf.* Fig. 1. In cross sections no orientation was observed. The spacings and orientations for new and old bone were the same. Bones from all different animals investigated gave the same x-ray diffraction pattern. It is interesting to note, that in calcified tendon from hen the mineral salts were oriented longitudinally (perpendicular to the small angle scattering of collagen).

When decalcifying the sections and taking new x-ray diffraction pictures the following phenomenon was observed. The 2.76 Å ring, which can be faintly seen after almost complete removal of the mineral salts, is oriented in longitudinal direction. The diffuse spacing of about 5–5.5 Å is oriented perpendicular to the 2.76 Å ring. Before decalcification these two rings showed no signs of orientation.

In rickets (dogs) no change in the diffraction picture in comparison with

[1] Too weak to be measured with accuracy.

normal bone could be detected. The orientation of the rings were the same as in normal material and were still very distinct.

Bones from dogs with hyperparathyroidism showed the same diffraction pattern as normal bone (7).

Preliminary electron micrographs of bones (replicas) showed a crystalline structure. Anisodiametric crystals, about 2×10^{-6} cm in width, were observed. The size of these crystallites agrees with the results obtained by x-ray methods. Details will be published later.

DISCUSSION

The results shown in Tables II and III indicate, that in case of calcium phosphates there can be relatively large variations in the composition of the mineral salts without being detectable by x-ray diffraction techniques. Therefore, it seems almost impossible to deduce the exact nature of the inorganic compounds in bone tissue by analysis of the x-ray diffraction pattern. As Haversian systems with different content of mineral salts, measured according to the method earlier described (1), show identical diffraction patterns, it also seems doubtful if it is possible to get information of the relative amounts of organic material present with this technique. This is in disagreement with the suggestions of Clark and Mrgudich (4).

Information about changes in the molecular structure of bone by x-ray diffractions therefore seems to be possible only when the orientation of the mineral salts is changed. The strong 2.76 Å ring, which originally is unoriented in longitudinal sections, becomes oriented when the specimen is almost completely decalcified.

X-ray micro-diffraction investigations of longitudinal sections of individual Haversian systems show, that under different conditions, such as absence of mechanical stress (6), rickets, and hyperparathyroidism no change in the orientation of the mineral salts takes place.

SUMMARY

X-ray micro-diffraction investigations of synthetic calcium phosphates of different composition and of bones show, that there can be relatively large variations in the chemical composition without changes in the interplanar spacings. The fact that the orientation of the mineral salts is the same in newly formed and old bone as well as in hyperparathyroidism, indicates that the ultrastructure of bone remains relatively unchanged in different physiological and pathological conditions.

REFERENCES

1. Amprino, R., and Engström, A., *J. Gen. Physiol.* (In press.)
2. Brandenberger, E., and Schinz, H. R., *Helv. Med. Acta*, **12**, Suppl. 16 (1945).
3. Chesley, R., *Rev. Sci. Instr.*, **18**, 422 (1947).
4. Clark, G. L., and Mrgudich, J. N., *Am. J. Physiol.*, **108**, 74 (1934).
5. Dallemagne, M. J., *Ann. Rev. Physiol.*, **12**, 101 (1950).
6. Engström, A., and Amprino, R., *Experientia*, **6**, 267 (1950).
7. Engström, A., and Engfeldt, B., *Acta Path. Microbiol. Scand.* (In press.)
8. Fankuchen, J., *Ann. Rev. Biochem.*, **14**, 207 (1945).
9. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
10. Kuyper, A. C., *J. Biol. Chem.*, **159**, 411 (1945).
11. —— *J. Biol. Chem.*, **159**, 417 (1945).
12. Mellanby, E., Experimental Rickets, Medical Research Council, London, 1921.
13. Pucher, G. W., Sherman, C. C., and Vickey, H. B., *J. Biol. Chem.*, **113**, 235 (1936).

BRIEF NOTES

THE RELATION OF METAPHOSPHATE FORMATION TO CELL DIVISION IN YEAST[1]

CARL C. LINDEGREN

Southern Illinois University, Carbondale, Illinois

Received August 7, 1950

The identification of the volutin (metachromatin) in yeasts as metaphosphate by Wiame (2) has facilitated the study of its physiological function. Lindegren (1) showed that when yeasts are grown in a medium rich in phosphate, the accumulation of metaphosphate on the chromosomes and nucleolus precedes budding, and he concluded that the synthesis of metaphosphate is essential to the division of the chromosomes. Observations of growing yeast cells have revealed that, in some cultures, budding cells do not show visible metaphosphate on the chromosomes. This fact has reopened the question and the present paper reports observations of the growth of yeast (increase in cell number and increase in the nitrogen content of cells) in synthetic and natural media containing different concentrations of phosphate. These experiments have shown that budding is invariably preceded by the deposition of metaphosphate on the chromosomes, even in very low concentrations of phosphate, but during the period of rapid budding the metaphosphate may disappear from the chromosomes if the initial concentration of phosphate is low. The maximal rate of division appears to depend upon the continued maintenance of metaphosphate on the chromosomes.

PROCEDURE

A 10 per cent suspension of Budweiser yeast was made up in distilled water and 0.5 ml added to 100 ml portion of medium in 250 ml Erlenmeyers. The culture was shaken at 30° C.

The synthetic medium used in these experiments had the following composition:

0.84 g K_2SO_4, 0.2 g $MgSO_4 \cdot KH_2O$, 0.074 g $CaCl_2$, 5.425 g $(NH_4)_2SO_4$, 4.0 mg Thiamine, 4.0 mg Pyridoxine, 4.0 mg Pantothenate, 1.0 mg Biotin, 0.2 mg PaBA, 0.2 mg Nicotinic Acid, 0.2 mg Inositol, 0.2 mg Riboflavin.

Four per cent dextrose was used. KH_2PO_4 level in our standard medium is 768 mgms per liter (175 mgm P per liter) which is in excess of the requirements. In these experiments the concentration, in terms of P per liter was varied from 0 to 100 mgms.

The natural medium contained: Peptone, 0.35 per cent; liquid yeast extract (Anheuser-Busch, Inc.), 0.2 per cent; dextrose, 4 per cent; and vitamins, as in the synthetic medium. The liquid yeast extract contains 8.25 mgm of phosphate P per ml.

The course of growth was followed by 1) cytological observations of metaphosphate in the cell, 2) cell counts, and 3) nitrogen determinations.

[1] This work was supported by grants from Anheuser-Busch, Inc. and The United States Public Health Service.

1) *Cytology:* A 3 to 5 ml sample of the suspension was centrifuged, decanted, 5 or 6 drops of metaphosphate stain added, and the cells examined under the microscope for metaphosphate. The stain contains 20 ml formalin saturated with toluidine blue, 8 ml water saturated with toluidine blue and 4 ml of 85 per cent lactic acid. The result was expressed as percentage of cells containing metaphosphate.

2) *Cell counts:* A 5 ml sample of the suspension is introduced into a 100 ml volumetric flask containing 1 ml of 5 per cent I_2 in 10 per cent KI solution, and diluted to 100 cc with distilled water. The iodine kills the cells until counts can be made. This suspension is counted in a haemocytometer and calculated on a cells-per-ml basis. The cells, disregarding buds, are counted first; then a count of cells which have buds attached is made. A total cell count is arbitrarily defined as cell count plus one-half the number of buds.

3) *Nitrogen determinations:* (Direct Nesslerization Method).

A 10 ml sample was centrifuged, washed once with distilled water, centrifuged and resuspended in 10 ml. It was found that a volume of suspension containing 10 to 150 micrograms of N (approximately 20 000 000 cells) was convenient for this analysis. This volume is introduced into an NPN tube containing exactly 1 ml of 18N H_2SO_4 (1:1), and digested until a brown color develops and heavy white fumes appear. Tubes are allowed to cool and two drops of 30 per cent H_2O_2 (superoxol) are added and the tube heated again until H_2O_2 decomposes. If the solution stays colorless, digestion may continue. If the solution turns brown again, more H_2O_2 is added, one drop at a time with heating until the solution remains colorless. After this point is reached, Hengar tubes are inserted in the mouths of the NPN tubes and digestion is continued for 30 minutes. Solutions are allowed to cool, diluted to 35 ml mark with distilled water, and Nessler's reagent (Bock-Benedict modification) is added to the 50 ml mark. Samples are read in the Klett-Summerson photoelectric colorimeter against a blank containing no nitrogen. A blue No. 42 filter is used. Standards are set up containing 50 and 100 micrograms of nitrogen per tube which read 90 and 180 respectively. The figures obtained from reading of samples are translated to micrograms per tube and per ml suspension.

EXPERIMENTAL RESULTS

Fig. 1 is a graph showing the total cell count (cells plus one-half buds) over a 12 hour period in media containing three different levels of P: 2.7, 5.4, and 10.8 mgm per liter. Phosphate is a limiting substance in this nutrient medium. About 35×10^6 cells per ml were produced in the 2.7 and 5.4 mgm tests, while nearly 50×10^6 cells were present in the 10.8 mgm test. The nitrogen per ml of washed suspension was 77, 86, and 110 micrograms in the three different levels of phosphate. Metaphosphate content of the cells rises to a maximum at about 2 hours after inoculation and drops to a low level after 4 hours in 2.7 and 5.4 mgm and 6 hours in 10.8 mgm of P; the metaphosphate content falls off during the period of rapid growth indicating that it is consumed during cell division.

Fig. 2 shows the effect of increasing the P from 10.8 mgm per liter to 91 mgm per liter. The optimal concentration lies around 23 mgm per liter. There is a rapid initial increase of metaphosphate on the chromosomes which is depleted during the period of rapid growth.

Fig. 3 shows the growth of yeast and the synthesis of nitrogen in natural medium. The concentration of P is corrected for that added in the liquid yeast extract. The growth rate and the synthesis of nitrogenous compounds are enormously increased. 27 mgm of P per liter are adequate to sustain an extremely rapid growth rate. In natural medium metaphosphate is maintained on the chromosomes much longer at the same level of phosphate in the medium, and does not drop nearly so low as in synthetic medium in spite of the fact that the depletion of the reserve is much more rapid, due to the more rapid growth rate.

Observations of yeast, during adaption to galactose, have revealed 1) that meta-

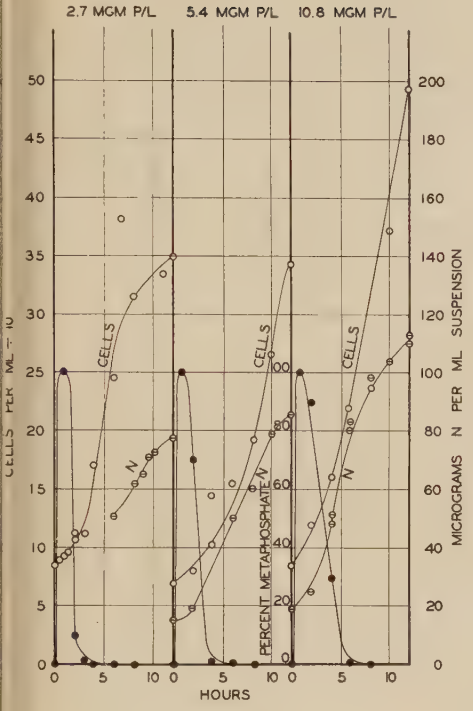


Fig. 1.

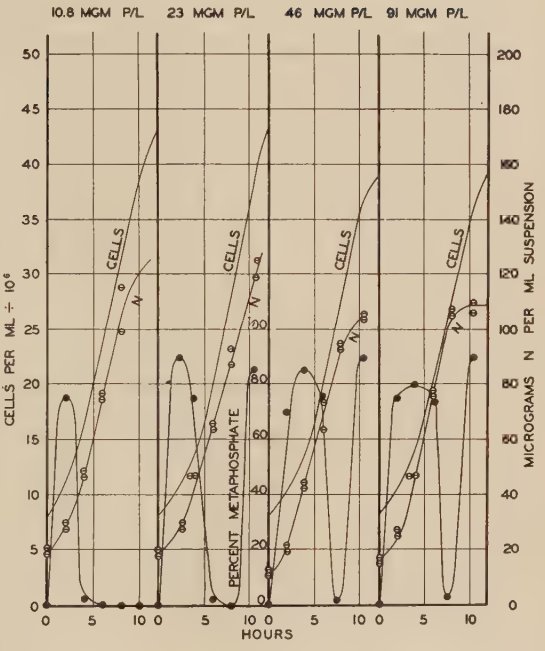


Fig. 2.

phosphate is not deposited heavily on the chromosomes until after some galactose has disappeared and 2) that the disappearance of phosphate from the nutrient medium ceases when the sugar is exhausted. These facts strengthen the view that metaphosphate serves as a source of energy in metabolism.

TABLE I

Number of Yeast cells after 18 hours in media containing different concentrations of phosphate.

Synthetic Medium		Natural Medium	
P mgm/L	Cells/ml/10^6	P mgm/L	Cells/ml/10^6
0.6	19.5		
2.7	30.5		
2.7	34.9		
5.5	44.4		
11.0	53.5	27	222.5
11.0	50.0	38	237.5
22.8	45.7	100	237.5
47	51.6		
94	53.0		
188	60.2		

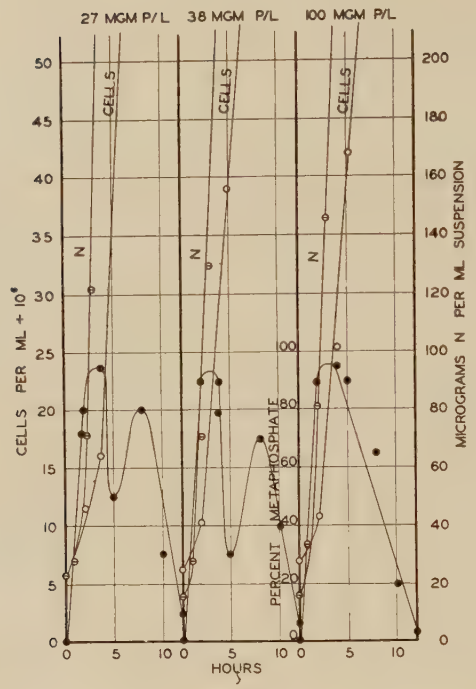


Fig. 3.

Table I shows the population after 18 hours in different media and different concentrations of P, in millions of cells per ml. There are more than four times as many cells in natural medium as in synthetic medium. Phosphate is limiting in the synthetic medium below 11 mgm per liter. About 38 is probably adequate in natural medium.

CONCLUSION

Phosphate is converted to metaphosphate by the yeast cell and appears on the chromosomes and nucleolus before budding can occur. The maintenance of metaphosphate on the chromosomes is essential for the attainment of the maximal growth rate. A slow growth is possible without detectable metaphosphate appearing in the cell, but this slow rate only occurs shortly after an initial deposit of metaphosphate has provided the necessary energy. In natural medium the increase in growth rate and production of cells is associated with the synthesis of large quantities of metaphosphate.

REFERENCES

1. Lindegren, Carl C., The Yeast Cell, Its Genetics and Cytology, Educational Publishers, Inc., St. Louis, 1949, 384 pp.
2. Wiame, J. M., *Bull. Soc. Chim. Biol.*, **28**, 552 (1946).

NEUE VERSUCHE ZUR LEPTONIK INTERMITOTISCHER ZELLKERNE

HANS H. PFEIFFER

Laboratorium für Polarisationsmikroskopie, Bremen, Germany

Received September 15, 1950

Allgemein wird angenommen, dass intermitotische (Ruhe-) Kerne von Pflanzenzellen im wesentlichen aufgebaut werden von Polypeptidketten, in deren Gefüge in wechselnden Mengen Nucleinsäuren von ausgesprochener Kettenstruktur eingebettet sind (3, 4, 12). Die innerhalb des Kerngefüges präformierten Chromosomen (6, 11) zeigen die beiden Komponenten längs ihrer Haupterstreckung, auch wenn das Verhältnis von Polypeptid- und Nucleinsäuremolekeln an den einzelnen Stellen variieren mag. Nach der an Kernen und Chromosomen beobachteten Doppelbrechung sind die Nucleinsäuremolekeln parallel zur Achse der Chromosomen angeordnet, aber „nothing is known concerning the orientation of the protein component" (1). Durch Anwendung der Ausstrichtechnik an Mikrosporenmutterzellen (MMZ) kann man aber zu einer wichtigen Entscheidung über die *leptonische Orientierung auch der Polypeptidketten* in intermitotischen Zellkernen gelangen.

Die Antheren junger Blüten von *Tradescantia, Hemerocallis* o. a. werden abgeschnitten und sofort schnellstens (innerhalb 3–5 Sek.) ohne Wasser zerquetscht und mit einem sauberen Skalpell über den Objektträger gestrichen. Vorgezogen werden Stadien *nach beginnendem Abrunden der MMZ* (nach Zerstörung des Archespors), doch gelingt gelegentlich auch ein Ausstrich von einem leidlich intakten Archespor. Bei den *Dehnungsversuchen* sind statt Objektträger aus Glas solche aus dilatablen, transparenten Aluminiumblättern nach R. V. Jones benutzt worden.

Die Digestionsversuche sind mit *Reiskleie-Nuclease* in einem $NaCH_3CO_2$- und CH_3CO_2H-Puffer (18) und die Kontrollversuche ohne das Enzym angestellt worden. Auf diese Weise wird in Kernausstrichen die Nucleinsäure eliminiert, so dass keine positive Feulgen-Reaktion mehr auftritt. Dass aber die Struktur der Polypeptidketten ziemlich unverändert bleibt, zeigt die Ausführung der Arginin-Reaktion (18) mit α-Naphthol und Harnstofflösung.

Zur *Imbition* der Objekte sind Mischungen von Cyclohexanol ($n_2 = 1{,}4645$), Benzylalkohol ($n_2 = 1{,}5376$), Zimtalkohol ($n_2 = 1, 5780$) und Zimtöl ($n_2 = 1, 6040$) benutzt worden.

Die optische Anisotropie ist, teilweise in verschiedenen Dehnungsgraden der Ausstriche, gemessen worden mit einem $\lambda/30$-*Glimmerkompensator* und dem *Halbschattenkeil* nach J. Macé de Lépinay (10^b, 14 a). Dieses Verfahren ist als höchst empfindlich nachgewiesen (8). Wenn auch die Fibrillen der Kernausstriche die Form von Zylindern zeigen, so wächst doch ihre Dicke so langsam mit zunehmender Entfernung vom Rande, dass ohne Bedenken die Beziehung für scheibenförmige Objekte mit planparallelen Grenzflächen:

$$\Delta n = \Gamma d,$$

angewandt werden kann, worin Γ die Retardation oder Phasendifferenz, d den Weg des Lichtstrahls im Objekt bedeuten.

Ausstrichversuche mit Zellkernen sind bereits von W. J. Schmidt (13, 14 b) von Thalassicolla u. a. beschrieben. Wie bei jenen Objekten tritt an ausgestrichenen Zellkernen von MMZ die Nucleinsäure durch den negativen Charakter der Doppelbrechung hervor. Wird das Deckglas über dem Ausstrich zwischen zwei Punkten hin und her bewegt, so bildet sich eine gallertige, fadenziehende Chromatinmasse, in welcher homogene, stark doppelbrechende Streifen auftreten. Die Anisotropie ist negativ in Bezug auf die Ausstrichrichtung, d. h. der Brechungskoeffizient des elektrischen Vektors parallel dieser Richtung ist kleiner als der Koeffizient des Vektors rechtwinklig dazu. Wird ein starker Scherdruck auf die eintrocknende Gallerte ausgeübt, so resultiert *fibrillare Differenzierung.* Die ganz glatten Fibrillen, deren Doppelbrechung negativ zu ihrer Länge ist, sind ohne mikroskopische Struktur. Die Intensität ihrer Anisotropie wird zu ungefähr $50 \cdot 10^{-3}$ gefunden, unterscheidet sich also von Werten an sonst Chromatin oder Nucleinsäure enthaltenden Objekten nicht.

Bei Behandlung mit digestierender Nuclease ist es möglich, die *Nucleinsäurekomponente fast* ohne Störung der Struktur *zu eliminieren.*[1] Dabei wird der negative Charakter der Doppelbrechung der Kernfibrillen reduziert oder bis zur Isotropie aufgehoben. Stets hängt die Intensität der Anisotropie von der Dauer der Einwirkung des digestiven Enzyms bezw. vom Digestionsgrade ab. Wird der Vers. mehr als 10–12 Std. fortgesetzt, so kann gar eine schwache positive Doppelbrechung (maximal: etwa $+5 \cdot 10^{-3}$) gefunden werden. Sicher sind diese Befunde mit der optischen Wirkung der parallel zueinander durch die ganze Länge der Fibrillen streichenden Polypeptidketten zu interpretieren.

Aber die Resultate mit den Imbibitionsversuchen sind noch mehr beweiskräftig. Die Fibrillen ausgestrichener Zellkerne der MMZ verhalten sich wie Wiener'sche *Stäbchenmischkörper*, d. h. wie Systeme isotroper (oder anisotroper) parallel gestellter Stäbchen vom Brechungskoeffizienten n_1 (klein im Vergleich zu der Wellenlänge des durchgelassenen Lichtes), welche in ein isotropes Medium der Refraktion n_2 eingebettet sind. Bezeichne n_e, n_0 den Brechungskoeffizienten des ausserordentlichen bezw. ordentlichen Lichtstrahles, δ_1, δ_2 die Partialvolumina der Stäbchen bezw. des Imbibitionsmediums ($\delta_1 + \delta_2 = 1$), so gilt nach Wiener für die Stäbchendoppelbrechung (10, 14):

$$n_e^2 - n_0^2 = \frac{\delta_1 \delta_2 (n_1^2 - n_2^2)^2}{(\delta_1 + 1) n_2^2 + \delta_1 n_1^2}.$$

Für das untersuchte Objekt kann man die Gl. besser modifizieren (16) zu:

$$n_e^2 - n_0^2 = \frac{\delta_1 \delta_2 (n_1^2 - n_2^2)^2}{2 [(\delta_1 + 1) n_2^2 + \delta_2 n_1^2] (\delta_1 n_1 + \delta_2 n_2)}.$$

Die *hauptsächlichen Folgerungen*, welche aus den Formeln zu ziehen sind (9, 10 a, 15), sind diese: Wenn die Doppelbrechung des Systems (vor und nach Einwirken der

[1] *Photometrische* Messungen an Kernausstrichen nach Färbungen (17) sind begonnen, aber noch nicht zu abschliessenden Ergebnissen gebracht worden.

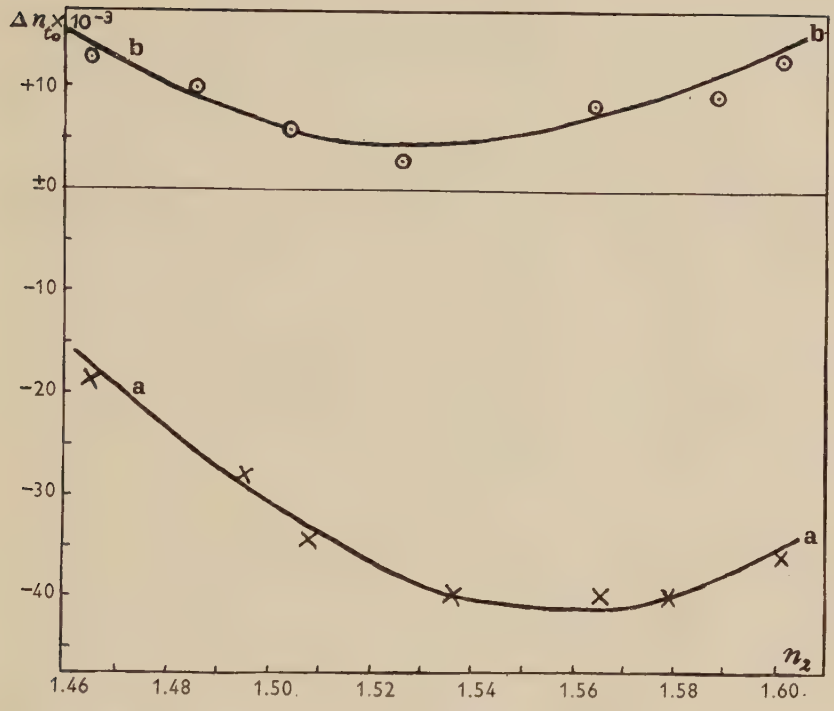


Fig. 1. Hyperbolische Kurven der (totalen) *Doppelbrechung als Funktion des Brechungskoeffizienten n_2 verschiedener Imbibitionsmedien;* a) mit ausgestrichenen Zellkernen ohne Einfluss des digestiven Enzyms (Doppelbrechung der Nucleinsäure-Komponente), b) nach Digestion (Anisotropie der Polypeptid-Ketten).

digestiven Nuclease) in einem Koordinatensystem gegen den Brechungskoeffizienten der Imbibitionsmedien aufgetragen wird, resultieren *hyperbolische Kurven*, deren Scheitel die Eigendoppelbrechung des Stäbchenmischkörpers bezeichnet, Fig. 1. Wenn die Partialvolumina δ_1, δ_2 der Stäbchen und der imbibierten Lücken des Mischkörpers gleich sind (also $\delta_1 = \delta_2 = 0.5$, d. h. $\delta_1\delta_2 = 0{,}25$), so erreicht dessen Doppelbrechung ein Maximum, und je mehr sich δ_2 dem Werte 0,5 nähert, um so mehr wächst die Steilheit der Kurve der Stäbchendoppelbrechung. Der Wert der Eigendoppelbrechung (also des Scheitels der hyperbolischen Kurven) in der am steilsten im Koordinatensystem verlaufenden Kurve der totalen Doppelbrechung beläuft sich gerade auf die Hälfte der Eigendoppelbrechung aller Stäbchen des Systems, Fig. 2.

Diese Folgerungen gelten indessen nicht mehr vollständig, wenn nur ein *bestimmter Anteil der micellaren Komponente* des Mischkörpers *orientiert* ist, d. h. wenn $\delta_1\delta_2 < 0{,}25$ (2, 9). Wenn durch Quellung des Mischkörpers hervorgerufene Desorientierungen micellarer Leptonen und die Mitwirkung eines photoelastischen Effekts (Spannungsdoppelbrechung) ausgeschlossen werden können, so lassen sich aus Lage und Verlauf der Kurven der totalen Doppelbrechung im Koordinatensystem *approximierte Werte*

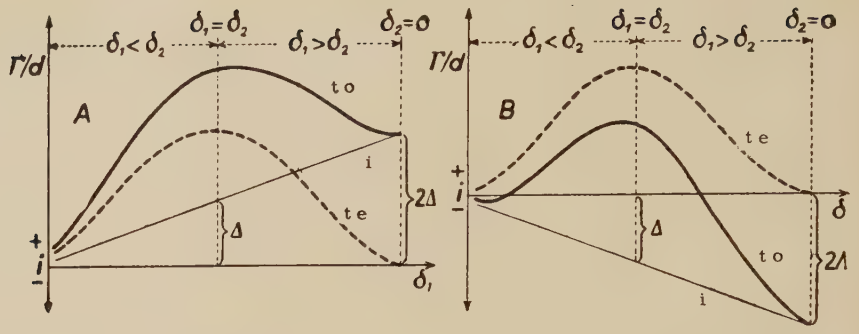


Fig. 2. *Einfluss der Verengerung der Lücken Wienerscher Mischkörper* auf die totale (to), Form- (te) und Eigendoppelbrechung (i) beim *Zusammenwirken von positiver Formdoppelbrechung mit* A) *positiver* Eigendoppelbrechung (Polypeptid-Ketten nach Digestion an den Kernausstrichen), B) *negativer Eigendoppelbrechung* (ohne das digestive Enzym). Ferner bezeichnet: i Gerade der Isotropie, δ_1, δ_2 Volumina der Textur (des Gefüges) bezw. der Lücken, Δ, 2 Δ Eigendoppelbrechung bei $\delta_1 = \delta_2$ bezw. $\delta_2 = 0$.

des Orientierungsgrades sowohl der Nucleinsäurekomponente (an nicht-digestierten Fibrillen) als auch der Polypeptidketten (nach Einfluss der Nuclease) ermitteln. So ergibt sich für die Nucleinsäuremolekeln $\delta_1\delta_2 = 0{,}072$ und ein Orientierungsgrad von fast 6 Prozent, für die Polypeptid-Komponente $\delta_1\delta_2 = 0{,}0608$ oder ein Orientierungsgrad von 3 bis 5 Prozent. Wie in rheologischen Versuchen mit Cytoplasma (7) überrascht die überaus *begrenzte Zahl der Leptonen*, welche durch das Dehnen (bezw. in den rheologischen Versuchen durch das Fliessen) *ausgerichtet* werden. Die polarisationsoptische Messung gestattet in solcher Weise die Ermittlung quantitativer Daten zur Abschätzung der Orientierung der Polypeptidketten ebenso wie der Nucleinsäure-Komponente der Kernausstriche (vgl. die in Tab. I zusammengestellten Resultate).

Endlich ergeben alle Kurven der Doppelbrechung in Abhängigkeit von *verschiedenen Dehnungsgraden* eine Kurvenschar von fast koinzidierendem Scheitel, aber variierender Steilheit, d. h. wechselnder Approximation an das Maximum von $\delta_1\delta_2$.

Tabelle I

Messungen mittels polarisierten Lichtes an ausgestrichenen Zellkernen von MMZ von *Hemerocallis* (Wellenlänge $\lambda = 5\,500$ Å).

	Nucleinsäure-Ketten	Polypeptid-Ketten
Leptonen	negativ	positiv
Eigen-Δn	$-43 \cdot 10^{-3}$	$+ 4{,}5 \cdot 10^{-3}$
Form-Δn ($n_2 = 1{,}515$)	$+ 9 \cdot 10^{-3}$	$+ 2 \cdot 10^{-3}$
$\delta_1\ \delta_2$	0,072	$0{,}060_8$
Orientierungsgrad (%)	5 ÷ 6	3 ÷ 5

Durch Vergleich der Werte dieses Produkts, welche aus variierenden Kurvenserien berechnet werden, kann gezeigt werden, dass die Beziehung zwischen den Orientierungsgraden der Leptonen und der beim Ausstreichen erzielten Dehnung der nuclearen Fibrillen durch eine Sättigungskurve ausgedrückt wird, welche beispielsweise mit einer Kurve der Beziehung zwischen der Länge der Leptonen und der Dauer von Hitzeeinwirkung (5) weitgehend übereinstimmt. Vielleicht ruft das Dehnen wie die Erhitzung bei jenem andern Phänomen eine Relaxation und einen Bruch intramolekularer Valenzbindungen hervor, macht innere molekulare Gruppen dem Versuchseingriff zugängig und bringt intermolekulare Bindungen zwischen den Molekeln zuwege. Die Entwicklung dieser beiden Bindungen scheint in beiden Fällen in *zwei stufenweise aufeinander folgenden Prozessen* stattzufinden, und diese abgestufte Ausbildung sekundärer Valenzen (van der Waals' Kohäsionskräfte) und primärer Valenzbindungen macht erneut einleuchtend, wie Dehnungen eine Tendenz zu krystallographischer Organisation verstärken.

LITERATUR

1. Bateman, J. B., in Rud. Höber, Physical chemistry of cells and tissues, Philadelphia & Toronto, Blakiston Co., 1945, S. 95, 185.
2. Bear, R. S., Schmitt, F. O., and Young, J. Z., *Proc. Roy. Soc. London* (B) **123**, 505 (1937).
3. Caspersson, T., Acidi nucleici, proteine e differenziamento normale e patologico, Torino, Ed. Rosenberg e Sellier, 1949, S. 53.
4. Frey-Wyssling, A., Submicroscopic morphology of protoplasm and its derivatives, New York & Amsterdam, Elsevier, 1948, S. 140.
5. Joly, M., *Kolloid-Z.*, **115**, 83 (1949).
6. Mirsky, A. E., and Ris, H., *J. Gen. Physiol.*, **31**, 1 und 7 (1947).
7. Pfeiffer, Hans H., *Nature*, **162**, 419 (1948), und unveröff. Vers.
8. —— *Optik*, **5**, 217 (1949).
9. —— *Z. Naturforsch.*, **4** (b), 307 (1949).
10. —— Das Polarisationsmikroskop als Messinstrument in Biologie und Medizin, 6. Braunschweig, Friedr. Vieweg & Sohn, 1949. — a) S. 7, 10. — b) S. 55, 64.
11. —— Experientia **6**, 334 (1950).
12. —— Submikroskopische Morphologie von Kern und Chromosomen, in A. Jores, Synopsis, Hamburg (in Druck).
13. Schmidt, W. J., *Arch. Protistenk.* **78**, 613 (1932).
14. —— Handb. biol. Arbeitsmeth., (V) **10**, 435, 479, 498 (1934). — a) S. 535. — b) S. 660.
15. —— *Verhandl. Zool. Ges.*, **41**, 307, 359, 363 (1939).
16. Schmitt, F. O., *J. Appl. Phys.*, **9**, 109 (1938).
17. Schrader, F., and Leuchtenberger, C., *Exptl. Cell Res.*, **1**, 421 (1950).
18. Serra, J. A., and Queiroz-Lopes, A., *Chromosoma* **2**, 576 (1944).

ACTION OF TRYPSIN ON THE EGGS OF DENDRASTER EXCENTRICUS

A. R. MOORE

Hopkins Marine Station, Pacific Grove, California, and the Physiological Laboratory, University of Portland, Portland, Oregon

Received November 15, 1950

In a study of the effects of trypsin on the unfertilized eggs of sea urchins, this writer has found that in the case of *Strongylocentrotus purpuratus* and *Str. drøbachiensis* the eggs under certain conditions were so injured by the trypsin that they could not be fertilized, nor give other outward sign of trypsin effect. Those which were fertilized after treatment with trypsin did not throw off fertilization membranes. In this respect their behavior is similar to that of *Psammechinus miliaris* described by Runnström (7) but differs from that of the eggs of *Echinocardium cordatum* in which the same author has reported that fertilization membranes form a result of treatment with proteolytic enzymes (7). These results have been confirmed with the eggs of *Dendraster excentricus*, using trypsin.

In the following experiments with *Dendraster* eggs crystalline trypsin was used. In practice the weighed charge of trypsin was dissolved in cold sea water, then filtered and the clear filtrate used as a stock solution, from which dilutions were made. Such solutions remained active for hours if kept at a temperature of 20° C or lower, but were inactivated by boiling. An observation was made by putting 5 cc of the required solution in a watch glass, a drop of eggs was introduced, the whole gently stirred for a moment and then observed with the low power objective. Almost at once, in the stronger solutions, membranes began to appear, in 30 seconds approximately in a solution of 3×10^{-5} M trypsin, and about 3 times that long in a 3×10^{-8} solution. Since crystalline trypsin was used in the experiments it is safe to assume that the results were due to that enzyme (2).

In order to make sure that the membranes observed were genuine fertilization membranes, the eggs were put to the test of artificial parthenogenesis (4). It was found that if the eggs, following membrane formation, were put into hypertonic sea water for 17–22 minutes at 20° C and then set to develop in sea water, normal segmentation occurred in a large percentage of the eggs. Therefore the membranes formed on the eggs of Dendraster as a result of the action of trypsin are fertilization membranes.

These facts have a significant bearing on the problem of enzyme action on cells. Now it is generally held that proteolytic enzymes do not digest living cells, but

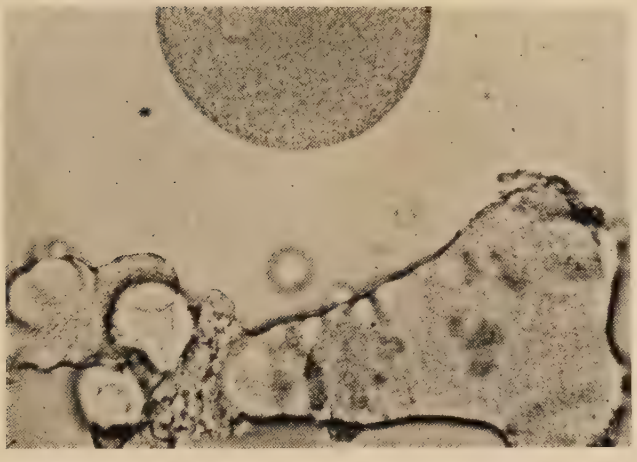


Fig. 1. Amoeboid form of unfertilized egg caused by action of trypsin 3×10^{-5} for three hours. In the upper field is shown a normal unfertilized egg for comparison.

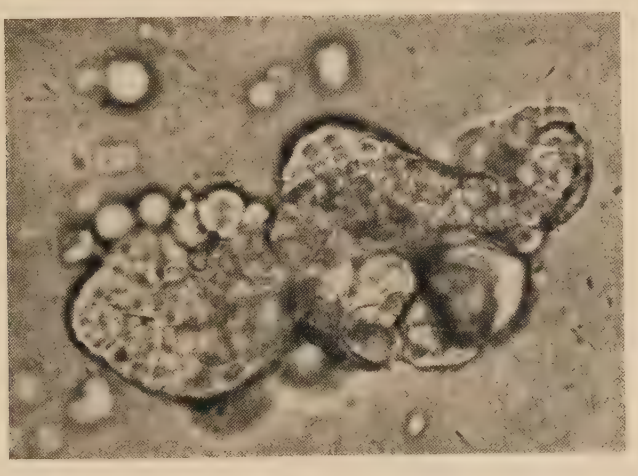


Fig. 2. Partially digested egg 4 hours after fertilization showing cellular masses, evidence that cytoplasm is not killed prior to the action of trypsin.

only dead ones (3, 5), with the possible exception that ficin digests living intestinal worms (6). Resistance on the part of the cell to enzymatic action may be due either to failure of the enzyme to enter the cell or, if it does enter, the enzyme may be rendered inactive by an inhibitor in the cytoplasm. The fact that trypsin causes such a deep-seated reaction as a membrane-formation is evidence that trypsin molecules penetrate the cortex, and as a result set off the reactions leading to membrane formation and the liberation of substances responsible osmotically for the entry of water through the membrane which is essential to its elevation.

Chymotrypsin caused rounding of the eggs, but in only rare instances were any membranes formed, nor was any evidence obtained of parthenogenetic effectiveness.

A further reaction of even more profound nature can be demonstrated in the eggs of *Dendraster* by long continued action of trypsin on the unfertilized egg. If the unfertilized eggs in which membranes have been formed by means of trypsin are allowed to remain in the solution for two or three hours, a large per cent of them undergo changes in form. First the membranes dissolve, then many of the eggs lose their spherical shape, flatten and become amoeboid (Fig. 1), eventually disintegrating. One possible explanation is that the surface action of the trypsin results first in the death of the egg and that only then is the latter attacked by the enzyme and broken down. That such is not the case is shown by the fact that if, when the eggs have been reduced to amoeboid form, they are transferred to sea water and fertilized some of them undergo regular cleavage in parts of the cytoplasm, forming irregular cellular masses (Fig. 2). This result makes clear that the intrinsic structure of the unfertilized living egg is attacked and partially broken down by trypsin.

The facts at hand, then, prove that trypsin enters the cortex of the unfertilized egg and initiates reactions leading to the formation and elevation of the membrane, and that trypsin breaks down the structure of the living egg, eventually digesting it.

In contrast to the unfertilized egg in its very labile nature with respect to the action of trypsin, the fertilized egg shows great stability, and, even when lying in a solution of trypsin, continues to segment apparently normally. How are we to account for the fact that fertilization abolishes the vulnerability to trypsin on the part of the egg? The fertilization membrane is permeable to trypsin since even in eggs long fertilized, trypsin passes through the outer membrane without weakening it and dissolves the hyaline layer which closely invests the egg. It is therefore hard to see why the enzyme should not enter the cytoplasm, not only because the molecules are in direct contact with the plasma membrane of the cell, but also for the reason that the permeability of the egg is increased by fertilization (1). There remains the possibility that inhibitor substances are formed or unmasked in the cytoplasm, following on fertilization, and that these render the trypsin molecules inactive.

The trypsin and chymotrypsin used in the foregoing experiments were kindly given me by Dr. M. Kunitz of the Rockefeller Institute, and I take pleasure in expressing my cordial thanks to him.

SUMMARY

Crystalline trypsin in 3×10^{-8} M concentration and higher, elicits fertilization membranes in the unfertilized eggs of *Dendraster excentricus*. These membranes are adequate in artificial parthenogenesis. If the action of trypsin on these eggs is continued for two or three hours the result is first, digestion of the membranes, followed later by reduction of the egg to amoeboid form. When fertilized, some of the partially digested eggs segment and form irregular cell masses, thus demonstrating that, in response to trypsin, there is first the cortical reaction giving rise to the fertilization membrane, and second, the progressive digestion and disintegration of the cytoplasm.

Chymotrypsin causes rounding of the unfertilized eggs and, in rare instances, a few membranes, but the enzyme is not an adequate parthenogenetic agent.

Fertilization of the egg renders the cytoplasm resistant to trypsin. The facts lead to the suggestion that fertilization liberates trypsin inhibitors in the cytoplasm.

REFERENCES

1. Jacobs, M. H., and Stewart, D. R., *J. Cellular Comp. Physiol.*, **7**, 333 (1936).
2. Jensen, AA. Bohus, *Nature*, **162**, 931 (1948).
3. —— *Exp. Cell Res.*, **1**, 351 (1950).
4. Loeb, J., Artificial Parthenogenesis, Chicago, 1913.
5. Northrop, J. H., *J. Gen. Physiol.*, **9**, 497 (1926) and **30**, 375 (1947).
6. Robbins, B. H., *J. Biol. Chem.*, **87**, 251 (1930).
7. Runnström, J., *Arkiv Zool.*, **40 A**, No. 17, *1* (1948).

THE MORPHOLOGY OF NEUROGLIAL NUCLEI IN THE CAT, ACCORDING TO SEX

MURRAY L. BARR

Department of Anatomy, University of Western Ontario, London, Canada

Received December 29, 1951

The purpose of this brief report is to point out a difference in the morphology of neuroglial cell nuclei in mature male and female cats. The sex difference is similar to that described previously for nerve cell nuclei (1, 3). To facilitate comparison, there follows a summary of the pertinent observations concerning the nerve cell nucleus.

In *female* cats, nerve cell nuclei contain a special mass of chromatin 0.7–1.0 μ in diameter. This nuclear component has been called a "nucleolar satellite," provisionally, since it is usually situated close to the much larger nucleolus. The nucleolar satellite contains desoxyribosenucleic acid, judging from a positive Feulgen reaction and an affinity for methyl green. In *male* cats, nerve cell nuclei lack a nucleolar satellite of comparable size. However, a definite nucleolar satellite appears in male nerve cells during periods of rapid synthesis of Nissl material (2). It is thought that male nerve cells contain a nucleolar satellite which, under more usual conditions, lies at the limit of resolution with the standard microscope.

Of the routine stains for nerve tissue, thionin (6) has been found especially useful for nuclear detail. The larger, paler neuroglial nuclei, often with an irregular outline, are taken to be those of astrocytes; smaller, darker and spherical nuclei are considered to be nuclei of oligodendrocytes. Numerous intermediate forms are encountered which are difficult to classify. Nuclei of *male* neuroglial cells (of both types) contain chromatin in the form of small particles scattered throughout the nucleus and pressed against the nuclear membrane (fig. 1, ♂). The larger nuclei often contain a single mass of greater size more or less centrally situated. In nuclei of *female* neuroglial cells (fig. 1, ♀) there is a special mass of chromatin, in addition to the nuclear constituents present in male cells. This sex-influenced body is located typically at the nuclear membrane. Its shape is plano-convex, being flattened on the membrane side. So far as can be judged by inspection, the sex-influenced chromatin mass of female neuroglial cells is of the same volume as the nucleolar satellite of female nerve cells. The supposition that these intranuclear structures are homologous in nerve cells and neuroglial cells gains support from staining reactions, since they are Feulgen-positive and have an affinity for methyl green in both instances.

Experiments which bring about an enlargement of the nucleolar satellite of male nerve cells (2), rendering it clearly visible with the standard microscope, are in-

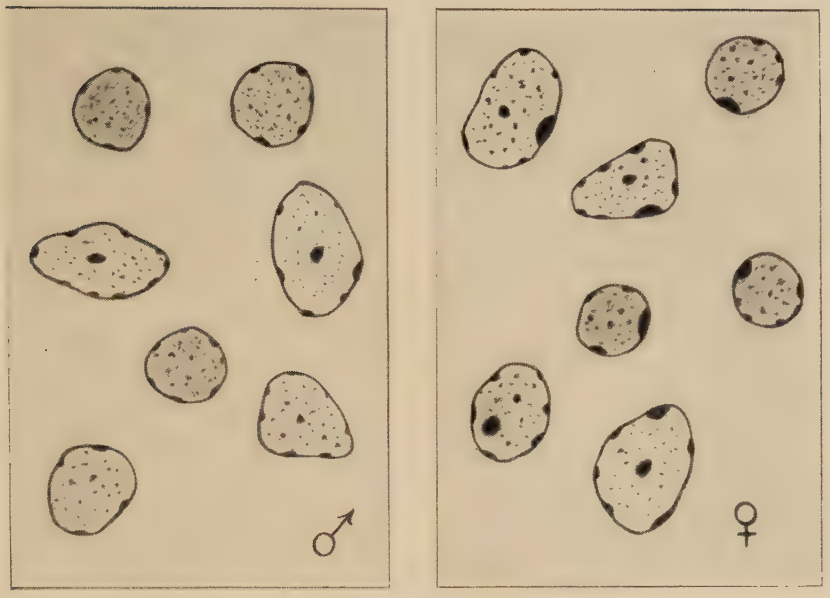


Fig. 1.

applicable to neuroglial cells. However, in a proportion of male neuroglial cells, there is an intranuclear body which, though smaller than the sex-influenced body of female nuclei, is rather larger and better defined than the chromatin particles generally. It is likely that in neuroglial cells, as in nerve cells, the sex difference in nuclear morphology is quantitative, resting on a difference in volume of an intranuclear structure.

The validity of the sex difference in neuroglial cell nuclei may be verified by a simple practical test. Thionin-stained sections of white matter from a number of animals can be sorted accurately, according to sex, by inspection of the neuroglial cell nuclei. Such sections, of course, contain no nerve cells or other clues concerning the sex of the animal from which a particular section was prepared.

If a similar sex difference is present in microglial nuclei, it is obscured by the numerous, large chromatin masses characteristic of these cells.

The presence of a sex-influenced body of comparable size in nerve cells and neuroglial cells is of interest with respect to the relation of the nucleolus and the sex-influenced body to each other and to the chromosomes. It is likely that the special nuclear structure under discussion is related to the X-chromosome, in view of the difference in volume according to sex (female 34 + XX, male 34 + XO (4)). Nerve cell nuclei contain a large nucleolus. Whether or not one subscribes to the generalization that neuroglial cells lack a nucleolus, there is certainly very little nucleolar material in neuroglial cells as compared with nerve cells. The great difference in volume of nucleolar material, in these two broad classes of cells, in the presence of a constant volume of sex-influenced chromatin lends support to the view that the

nucleolus and the sex-influenced intranuclear structure are related to different chromosomes (3). This speculation illustrates the need for studies on chromosome morphology in the cat and other species, similar to that reported for man (5) which showed the nucleolar chromosome to be an autosome.

The demonstration of a sex difference in the morphology of small nuclei lacking the large nucleoli typical of nerve cells encourages the search for a similar feature in somatic cells generally, an investigation currently in progress in this laboratory.

This study was supported by a grant-in-aid from the National Research Council of Canada.

REFERENCES

1. Barr, M. L., and Bertram, E. G., *Nature*, **163**, 676 (1949).
2. —— (in press).
3. Barr, M. L., Bertram, L. F., and Lindsay, H. A., *Anat. Rec*, **107**, 283 (1950).
4. de Winiwarter, H., *Arch. biol.*, Paris, **30**, 1 (1920).
5. Schultz, J., and St. Lawrence, R., *J. Heredity*, **40**, 31 (1949).
6. Windle, W. F., Rhines, R., and Rankin, J., *Stain Technol.*, **18**, 77 (1943).

DEVELOPMENT OF A SPECTRUM OF ASCITES TUMORS

GEORGE KLEIN

Institute for Cancer Research, Philadelphia, Pa., and Wallenberg Laboratory for Experimental Cytology, Institute for Cell Research, Karolinska Institutet, Stockholm, Sweden[1]

Received February 2, 1951

The fact that different malignant tumors show greatly different behavior with respect to their biological characteristics is well recognized today. Because of this diversity it has become necessary to work with a variety of tumors in most fields of experimental oncology, if any consequences of general validity are to be drawn. This is especially true for the field of experimental chemotherapy, as different tumors may show widely different reactions to the chemical agents tested. The necessity of using a broad variety of different tumors for chemotherapeutic studies has been recognized by many groups of workers. Thus, for instance, Stock's group reported the use of a "spectrum" of solid mouse and rat tumors for in vivo studies of tumor inhibition with different compounds (8).

The Ehrlich ascites tumor of the mouse has been shown to present several advantages over solid tumors for quantitative studies on the growth and biochemistry of neoplastic cells (3). Also, it has been successfully employed for the testing of different chemicals with possible tumor-inhibiting potency and for cytological studies (3, 5, 6, 7; for other ref. see (3). The availability of a homogeneous suspension of free tumor cells in the peritoneal fluid presents a uniform material, if compared to corresponding solid tumors (1) and makes possible determinations of average amounts of biologically important substances per cell. Furthermore, a known number of viable tumor cells could be used for inoculation, and tumor cells could be obtained and studied during the whole course of tumor development.

On the basis of these and other advantages of the "ascites tumor" form of growth, it was considered to be of great interest to try to transform a number of different solid into ascites tumors. In a previous study (4), a solid transplantable carcinoma of the mouse could be transformed in this way. It could be shown that during subsequent intraperitoneal transfers of the exudate produced by the i.p. inoculation of dissociated solid tumor masses, a gradual increase in the absolute and relative frequency of tumor cells in the fluid could be observed until finally the nearly "pure culture" state of tumor cells in the ascitic fluid could be reached. On the basis of this experience, the development of a spectrum of ascites tumors was attempted. The choice of solid tumors for the present experiment was guided by two criteria: the necessity of having a broad variety of morphologically and biologically different tumors and the desirability of working, in so far as possible, with tumors in inbred strains of mice.

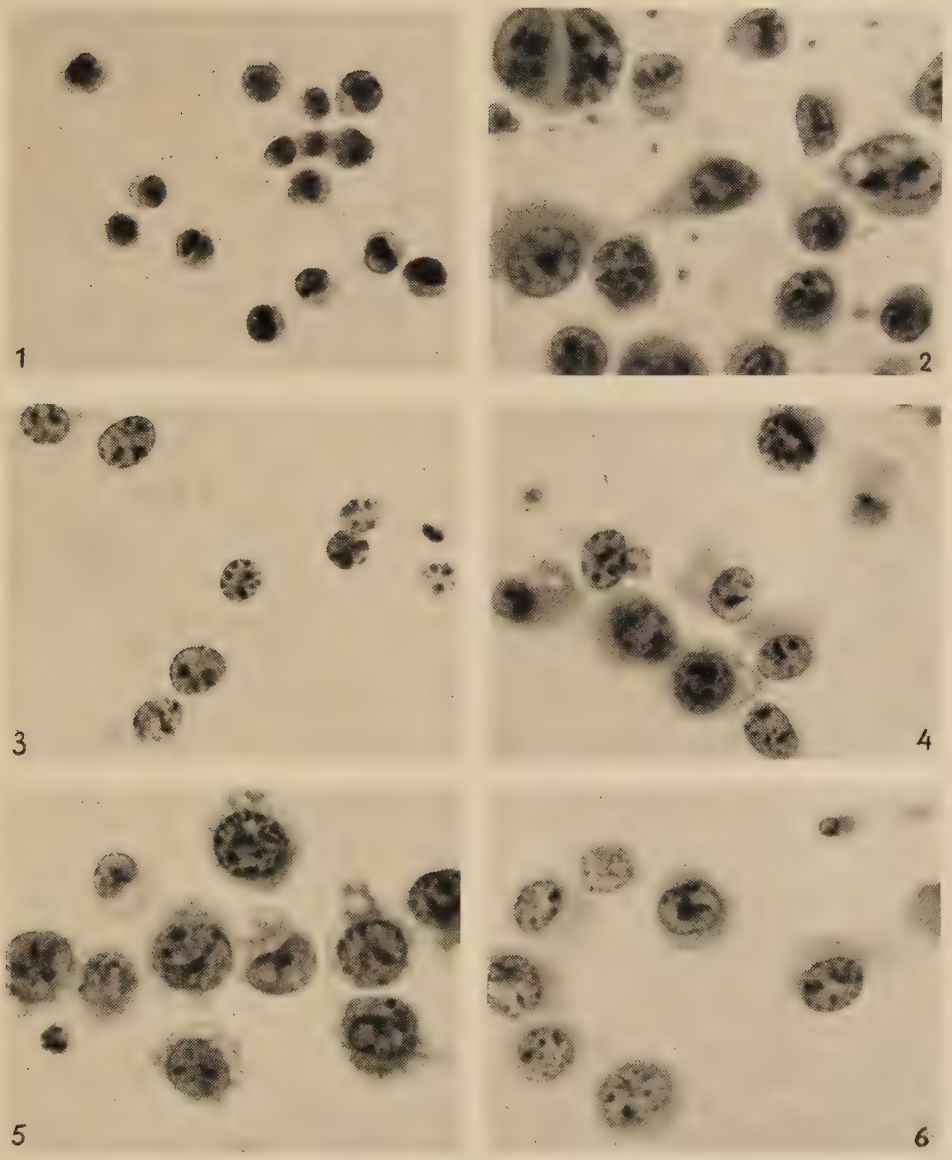
[1] Present address: Karolinska Institutet, Stockholm 60, Sweden.

TABLE I

List of tumors used for intraperitoneal inoculation and results.

No.	Name	Mouse Strain	Nature (tr: transplanted, sp: spontaneous)	Source	Result of i.p. inoculation (S: only solid growth, E: exudate)	Per cent tumor cells detected in exudate if formed	Transmission of tumor with exudate	No. of transplant generations with exudate alone	Median survival time in days on last transfer with 0.4 cc exudate	Per cent tumor cells in ascitic fluid on last transplant generation at median survival time	Note
1.	6C3HED	C3H	tr. lymphosarcoma	JML	E	48	+	12	10	93	
2.	—	dba	tr. lymphosarcoma	NCI	E	73	+	9	11	96	
3.	PLS	AKM	tr. lymphosarcoma	SKI	E	80	+	4	7	95	
4.	SPAD	A	sp. mammary adenoca.	ICR	E	0	+	3	55	1.2	
5.	SPAH	A	sp. mammary adenoca.	ICR	E	0	+	3	65	2	
6.	SPC3H	C3H	sp. mammary adenoca.	ICR	E	0	+	2	76	0	
7.	SPdba	dba	sp. mammary adenoca.	ICR	E	0	—				
8.	TA3	A	tr. mammary adenoca.	ICR	E	0	+	12	13	42[1]	
9.	ICR	C3H	tr. mammary adenoca.	ICR	E	0	—				
10.	EO771	C57bl.	tr. mammary adenoca.	SKI	E	0	+	4	33	3.5	
11.	15091a	A	tr. spindlecellca.	JML	E	10	+	8	13	70	
12.	S91	dba	tr. melanoma	ICR	E	0	—				
13.	H–P	nonspec.	tr. melanoma	SKI	S						
14.	Krebs 2	nonspec.	tr. ca. simplex	Aarhus	E	0	+	23	6	85	Ref. 4
15.	C1300	A	tr. neuroblastoma	JML	E	1	+	4	28	12	
16.	C954	C57 leaden	tr. hepatic ca.	JML	E	2.5	+	4	25	21	
17.	T241	C57bl.	tr. sarcoma	SKI	E	0	+	8	23	4	
18.	Sc37	nonspec.	tr. sarcoma	ICR	E	40	+	11	10	53	
19.	MC1A	C3H	tr. rhabdomyosc.	ICR	E	8	+	8	20	23	
20.	MC1E	C3H	tr. fibrosc.	ICR	E	0	+	5	19	12	
21.	MC1M	C3H	tr. sarcoma	ICR	E	0	+	12	19	54	
22.	MC1N	C3H	tr. sarcoma	ICR	S						
23.	Sc180	nonspec.	tr. sarcoma	ICR	S						
24.	WOS	AKM	tr. osteogenic sarcoma	SKI	E	0	—				
25.	—	C3H	tr. hepatoma	NCI	S						

All figures denoting tumor cell frequency are means of samples from 5–15 animals. Under "source" the laboratory is denoted from where the tumor actually was obtained. ICR: Institute for Cancer Research, Philadelphia; NCI: National Cancer Institute;



Figs. 1–6. Papanicolaou smears of ascitic fluids.
Fig. 1. 6C3HED lymphosarcoma, 6th transplant generation of ascites. 700×.
Fig. 2. 15091 a spindlecell ca., 7th transplant generation of ascites. 700×.
Fig. 3. C 1300 neuroblastoma, 4th transplant generation of ascites. 700×.
Fig. 4. MC1A rhabdomyosarcoma, 7th transplant generation of ascites. 700×.
Fig. 5. Krebs 2 solid ca., 18th transplant generation of ascites. 700×.
Fig. 6. MC1M sarcoma, 10th transplant generation of ascites. 700×.

Table 1 shows the list of the different tumors used. All tumors were thoroughly minced, mixed with saline and injected intraperitoneally in large amounts. If an exudate was produced after the tumor had developed in the peritoneal cavity, this exudate was used for further i.p. inoculation. Similarly, in all subsequent transplant generations, always the exudate, if any, was transferred.

A general tendency towards increasing frequencies of tumor cells in the ascitic fluids (as differentiated by the method of Papanicolaou) could be observed during the subsequent transfers with most of the exudate-producing tumors. This is in conformity with our previous observations on the Krebs 2 tumor (4), and is probably due to the selection by the method used of the particular tumor cells which are able to live in the fluid medium of the peritoneal exudate. In order to test whether or not these cells represent a morphologically distinguishable particular cell type, subcutaneous injections of the exudates were performed on each new transplant generation with all tumors. The morphological picture of the thereby produced solid tumors was then compared with that of the corresponding original solid tumor. In no cases have any relevant changes in the structure or type of the tumors been detected.

During the course of these experiments the author received (through the courtesy of Dr. Goldie) a manuscript of a paper in press of Goldie and Felix (2) about the growth of free tumor cells in the peritoneal fluid. The two tumors used by Goldie and Felix (Nos. 2 and 18 in Table 1) have also been included in the list of the tumors tested, and growth in the ascitic fluid could be obtained with both of them, in conformity with the findings of Goldie and Felix.

Figs. 1–6 show Papanicolaou smears of some of the ascitic fluids, where the concentration of tumor cells is relatively high.

The tumors with the high frequencies of tumor cells in the ascitic fluid, together with the Ehrlich and Yoshida ascites tumors, are to be used as an ascites tumor "spectrum" for biochemical and chemotherapeutic studies. Experiments with all tumors are being continued and the results will be reported later in detail.

This work has been carried out on a grant from Knut and Alice Wallenbergs Foundation. I wish to express my gratitude to Dr. T. S. Hauschka and Dr. J. Schultz for their continuous interest in this investigation and their most valuable advice and criticism.

REFERENCES

1. Goldberg, L., Klein, E., and Klein, G., *Exp. Cell Res.*, **1**, 543 (1950).
2. Goldie, H., and Felix, M. D., *Cancer Research.* (In press.)
3. Klein, G., *Cancer*, **3**, 1052 (1950).
4. Klein, G., and Klein, E., *Cancer Research.* (In press.)
5. Lettré, H., *Z. physiol. Chem.*, **268**, 59 (1941).
6. —— *Z. Krebsforsch.*, **57**, 1 (1950).
7. Seeger, P. G., *Arch. exp. Zellforsch.*, **22**, 356 (1939).
8. Stock, C. C., Sugiura, K., Dobriner, K., and Rhoads, C. P., Fifth International Congress of Cancer, Paris, 1950.

ELECTRON MICROSCOPE OBSERVATION ON THE FINE STRUCTURE OF BACTERIAL FLAGELLA

E. DE ROBERTIS and C. M. FRANCHI

Department of Cell Ultrastructure, Instituto de Investigación de Ciencias Biológicas, Montevideo, Uruguay

Received February 25, 1951

Recent work has focussed attention on the study of bacterial flagella from the chemical, structural, and functional point of view (1, 2, 4, 5). Weibull (5) purified flagella from *Proteus vulgaris* and studied some of their chemical and physicochemical properties reaching the conclusion that they are of protein nature. X-ray diffraction examination of flagella gave a wide angle pattern similar to that of keratin, myosin and the rest of this group of fibrous proteins (1). In this work it was suggested that flagella might correspond to the *contractile units* of muscle tissue and that they may represent *monomolecular muscles.*

The flagellar contraction has been investigated by Flemming *et al.* (2) on bacteria grown in low concentration of penicillin so as to inhibit cell division but not to impair cell growth; the rate of contraction was found to be influenced by "the stimulus of very small changes in radiant heat."

Electron-microscopically an exhaustive work was carried out on the flagellation of a large variety of bacterial species by Houwink and Van Iterson (4). In this work and in the earlier literature, flagella are generally considered to be homogeneous filaments. The authors mention however "faint indications of a cross structure" in some electron microphotographs.

In the course of an investigation on the action of crystalline trypsin (Armour) on elastic tissue (3) coiled filaments were observed which originated by fragmentation of flagella from a bacteria contaminating the trypsin. This bacteria studied at the "Instituto di Higiene Experimental of Montevideo" by Mr. Assandri proved to be a sporulated aerobic *Bacillus* with abundant peritrichy flagellation.[1]

Fig. 1 *a* shows two flagella of bacteria grown for 11 hours at 38° C in trypsin dissolved in the concentration of 0.1 per cent in phosphate buffer of pH 7. They appear to have a composite constitution with a central filament and a peripheral sheat. The filament about 120–150 Å wide makes a definite protrusion on the surface and shows indications of a coiled structure. The sheath which is best seen when the shadow is parallel to the flagellum makes very little protrusion and appears to be a very thin membrane. Few flagella are more or less intact as those of Fig. 1, in most

[1] Since the presentation of the manuscript this *Bacillus* has been identified by Mr. Assandri as *Bacillus Brevis.*

electron micrographs only products of the fragmentation of flagella can be seen as short filaments having a definite coiling (Fig. 2). Some of the filaments show rests of the surrounding sheath but most of them seem completely naked. The pitch of the coil varies from one filament to another.

Fig. 3 shows flagella (some still attached to a bacillus) in which the spiral fine structure of the naked filament is clearly seen. The pitch of the coil varies in different regions of the same flagellum. The minimal distance between the coils which can be measured in region marked *a* is of 80 Å, while in region *b* of the other flagellum is of 125 Å. Longer periods are also observed. Intact flagella isolated from *Proteous vulgaris* X19 with the technique described by Weibull (5) also showed indications of a spiral structure.

These preliminary observations suggest that the bacterial flagella are composite structures with a central trypsin-resistant coiled filament and a peripheral sheath which is probably digested by this enzyme. Observations in course demonstrate that the fibrous portion, which is probably endowed with the contractile function of the flagellum, is a tightly coiled double helix constituted by two interlacing filaments. A study of some physicochemical properties of this fibrous component of the flagella is being carried on and the results will be reported later.

REFERENCES

1. Astbury, W. T., and Weibull, C., *Nature*, **163**, 280 (1949).
2. Fleming, A., *J. Gen. Microbiol.*, **4**, 457 (1950).
3. Franchi, C. M., and De Robertis, E. (In press.)
4. Houwink, A. L., and Van Iterson, W., *Biochim. et Biophys, Acta*, **5**, 10 (1950).
5. Weibull, C., *Biochim. et Biophys. Acta*, **2**, 351 (1948).

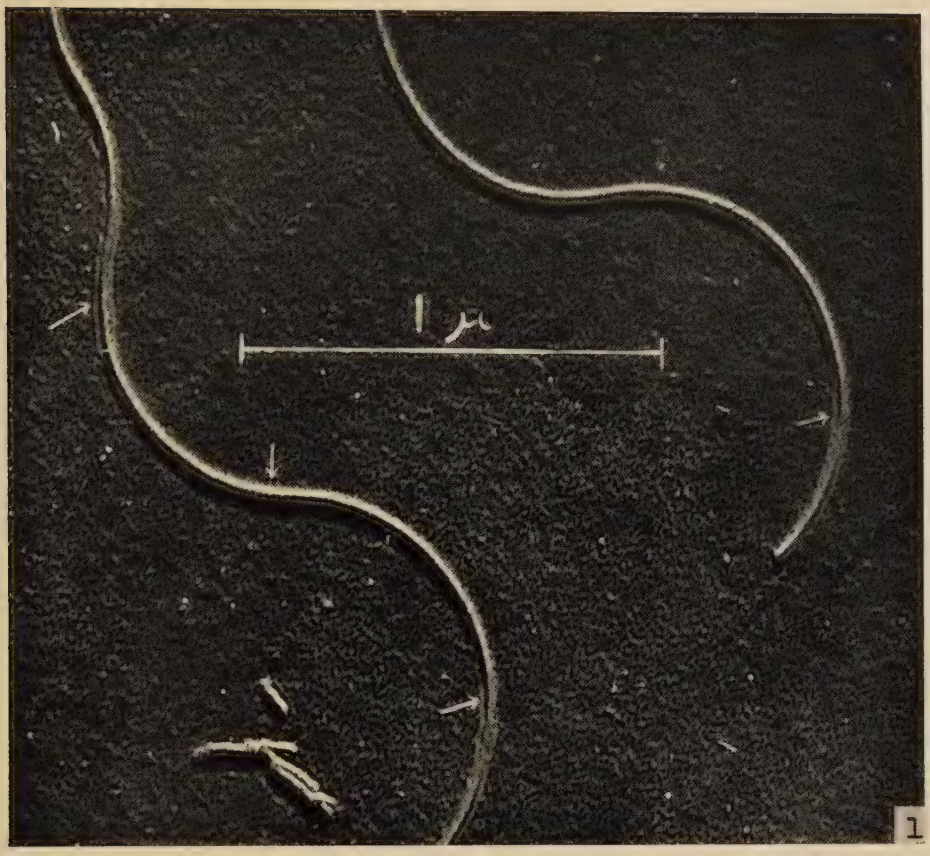


Fig. 1. Two Flagella showing a central coiled filament and a peripheral sheath. A few flagellar fragment is also present. Mag. 55 000 ×.

Fig. 2. Flagellar fragments showing a coiled structure with varying pitch and some rests of the sheath. Mag. 55 000 ×.

In both figures arrows pointing downward indicate places in which coiling is best observed and arrows pointing upward regions in which a peripheral sheath is seen.

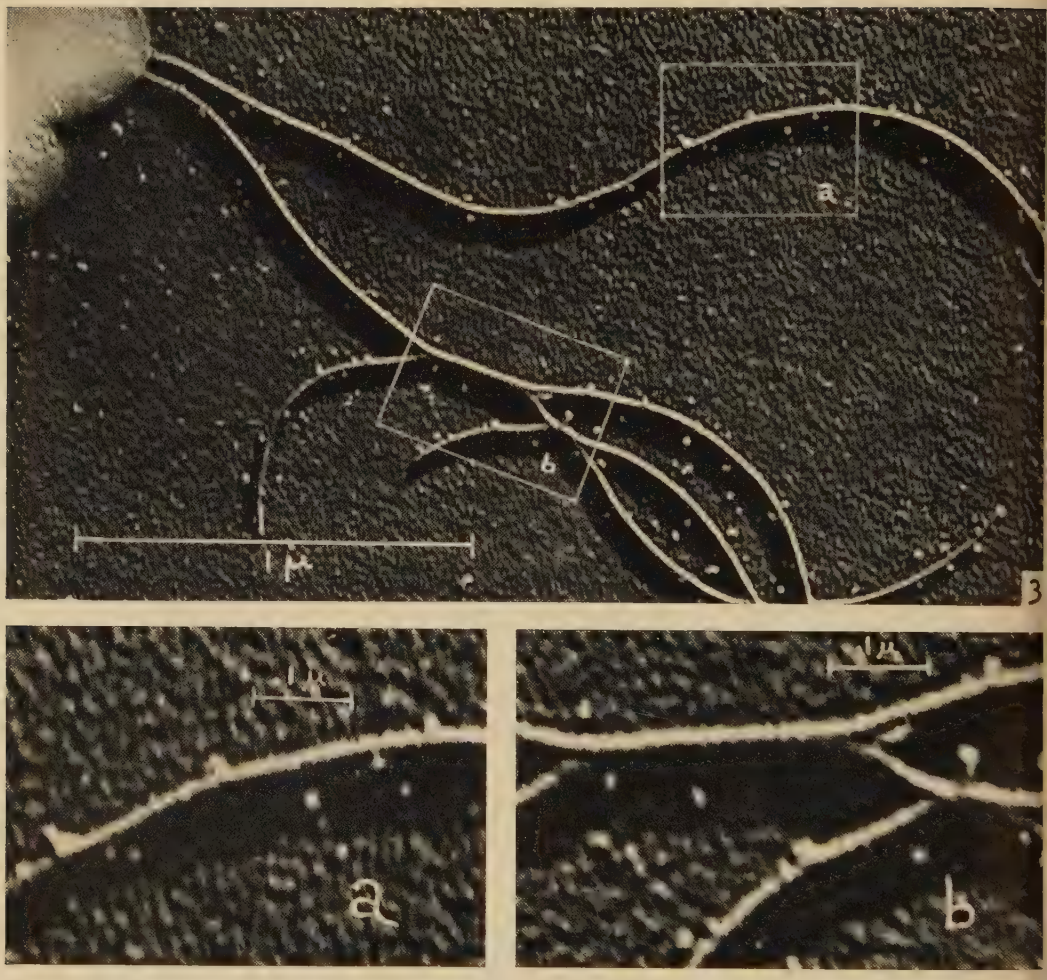


Fig. 3. Two flagella still attached to a bacillus and free flagella showing a spiral fine structure. The coiled filaments seem to be entirely naked, probably by digestion of the peripheral sheath. Mag. 55 000 ×.

Inserts *a* and *b* show the spiral structure of two regions of the flagella at a magnification of 130 000 ×. In *a* the pitch of the coil is of about 80 Å and in *b* of 125 Å.

All figures are from bacilli grown in 0.1 per cent crystalline trypsin (Armour) in phosphate buffer of pH 7 for 11 hours, purified by repeated centrifugations at 20 000 R.P.M. and washings in distilled water of pH 7. Specimens shadowed with palladium at an angle of 11° and observed with an EMU—2 C R.C.A. electron microscope.

MODIFIED SEMI-MICRO- AND MICRO-WARING BLENDORS FOR LOW TEMPERATURE USE[1]

SAM SOROF and PHILIP P. COHEN

Laboratory of Physiological Chemistry, University of Wisconsin, Madison, Wisc.

Received March 7, 1951

In the course of studies on the properties of liver proteins, the need arose for a semi-micro Waring blendor to be used for relatively long periods of time with the maintenance of temperatures close to 0° C. The following two adaptations of the commercially available semi-micro Waring blendor have proved satisfactory.

For use with 25 to 50 ml. volumes, the modification (Fig. 1) consisted of a metallic jacket soldered on to the base of the monel metal semi-micro blendor bowl for the purpose of containing ice-water or ice-saline mixtures. The apparatus was used in a cold room maintained at 4° C. The speed of the blending motor without the jar and with only the load of a tachometer was 13 000 r.p.m. An ice-water mixture stirred in the jacket maintained the temperature of the blended suspension below + 3° C during fifteen minutes of operation. Subfreezing temperatures were realized with the use of ice-saline mixtures under the same conditions.

For use with 5 to 15 ml. volumes, the additional modifications shown in Fig. 2 were devised. In this case, the ice-water or ice-saline mixture was contained in both outer compartments. The innermost chamber consisted of a stainless steel tube soldered to a modified bearing holder of the commercial stainless steel blade assembly. This micro-compartment was removable by unscrewing the nut at the underside of the base. Substitution of this cutting assembly and attached tube for the standard blade assembly permitted use as in Fig. 1. Similar performance was experienced in preliminary trials as with that described for the setup of Fig. 1.

The authors wish to thank Mr John Hipple for his technical suggestions and skillful construction of both pieces of apparatus.

---

[1] Aided in part by research grants from the Wisconsin Alumni Research Foundation, and the National Cancer Institute, National Institute of Health, Public Health Service.

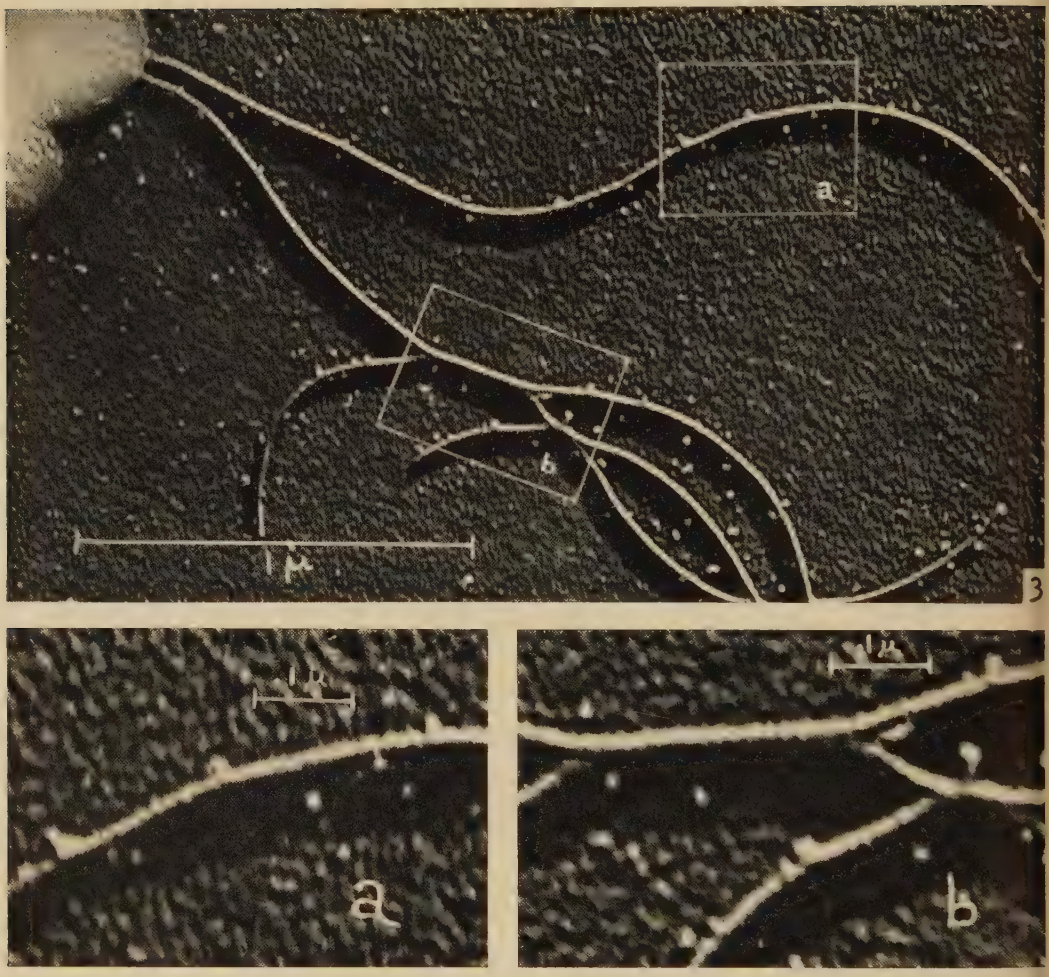


Fig. 3. Two flagella still attached to a bacillus and free flagella showing a spiral fine structure. The coiled filaments seem to be entirely naked, probably by digestion of the peripheral sheath. Mag. 55 000 ×.

Inserts *a* and *b* show the spiral structure of two regions of the flagella at a magnification of 130 000 ×. In *a* the pitch of the coil is of about 80 Å and in *b* of 125 Å.

All figures are from bacilli grown in 0.1 per cent crystalline trypsin (Armour) in phosphate buffer of pH 7 for 11 hours, purified by repeated centrifugations at 20 000 R.P.M. and washings in distilled water of pH 7. Specimens shadowed with palladium at an angle of 11° and observed with an EMU—2 C R.C.A. electron microscope.

MODIFIED SEMI-MICRO- AND MICRO-WARING BLENDORS FOR LOW TEMPERATURE USE[1]

SAM SOROF and PHILIP P. COHEN

Laboratory of Physiological Chemistry, University of Wisconsin, Madison, Wisc.

Received March 7, 1951

In the course of studies on the properties of liver proteins, the need arose for a semi-micro Waring blendor to be used for relatively long periods of time with the maintenance of temperatures close to 0° C. The following two adaptations of the commercially available semi-micro Waring blendor have proved satisfactory.

For use with 25 to 50 ml. volumes, the modification (Fig. 1) consisted of a metallic jacket soldered on to the base of the monel metal semi-micro blendor bowl for the purpose of containing ice-water or ice-saline mixtures. The apparatus was used in a cold room maintained at 4° C. The speed of the blending motor without the jar and with only the load of a tachometer was 13 000 r.p.m. An ice-water mixture stirred in the jacket maintained the temperature of the blended suspension below + 3° C during fifteen minutes of operation. Subfreezing temperatures were realized with the use of ice-saline mixtures under the same conditions.

For use with 5 to 15 ml. volumes, the additional modifications shown in Fig. 2 were devised. In this case, the ice-water or ice-saline mixture was contained in both outer compartments. The innermost chamber consisted of a stainless steel tube soldered to a modified bearing holder of the commercial stainless steel blade assembly. This micro-compartment was removable by unscrewing the nut at the underside of the base. Substitution of this cutting assembly and attached tube for the standard blade assembly permitted use as in Fig. 1. Similar performance was experienced in preliminary trials as with that described for the setup of Fig. 1.

The authors wish to thank Mr John Hipple for his technical suggestions and skillful construction of both pieces of apparatus.

---

[1] Aided in part by research grants from the Wisconsin Alumni Research Foundation, and the National Cancer Institute, National Institute of Health, Public Health Service.

Therefore the instrument described below has been developed. The working time is in this instrument reduced about one hundred times, thus making it possible to get significant data on the total absorption of whole inhomogeneous cells even on cell numbers of statistical validity in a relatively short period.

In the universal microspectrograph, earlier described in this journal (1) the object to be measured is placed on an objectholder, suspended by crystalline quartz plates. The holder is mechanically connected with the membrane of a mercuryfilled membrane chamber (2). A tube from this chamber dips into a mercuryfilled container, the level of which can be changed by aid of a reversible motor with continuously regulable speed. The movement of this container is electrically coupled with the paper-movement in a Speedomax type X-Y-recording potentiometer. In average measurements 1 μ object movement corresponds to 1 cm paper movement. The paper follows the object movement with a reproducibility of 0.2 μ independent of speed and direction of the movement.

The optical system projects an area of 0.4 μ^2 (numerical aperture 0.85) close to the optical axis of the system into a photomultiplier tube. If desired, higher lens apertures and correspondingly smaller fields can be used. The beam of light is split before it enters the microscope and the deviated ray hits another photomultiplier tube. By aid of an electronic ratio recorder (constructed by G. Lomakka) the pen on the X-Y-recorder writes the ratio between transmitted and incident light, independent of the often considerable variations in the mercury lamp used. The reproducibility of the transmission measurements in individual points is better than 2 per cent even with light source variations of $\pm$ 20 per cent. Measurements can be made in any wavelength of the continuous background of the mercury super high pressure arc (run under different loads in different wavelength ranges) down to 2 600 Å, and furthermore at 2 570 Å, 2 535 Å, 2 480 Å and with lower accuracy at 2 350 Å. Time for an individual run is 30 seconds. Compared with earlier photographic methods this means a great increase in accuracy and a great saving in working time. Additional time is gained by making the replotting of the curves in an automatic extinction calculator and integrator. In this instrument the transmission curve is placed on a moving table and the operator follows the curve with a pointer. The latter is coupled with a logarithmic potentiometer device giving impulses proportional to the corresponding logarithmic extinction coefficient, which is recorded on a separate recording 2-point writer, the paper of the latter moving synchronously with that of the transmission curve. Simultaneously an integrator arrangement registers continuously the surface under the extinction curve, that is the total extinction along the track.

The instrument has proved to work reliably in routine work and the desired decrease in working time compared with the earlier photographic methods has been achieved.

With a simple change the instrument can be used also for automatic recording of the absorption spectrum of an individual measuring point. In this case the movement of the paper is coupled with the wavelength drum of the monochromator and achromatic optics are used. This arrangement works efficiently down to 3 300 Å. Below that wavelength reliable measurements can as yet be made only of areas in a cell of the size of several square μ because of the lack of a truly continuous source of light with enough high specific intensity.

Since this new scanning device works about 100 times faster than previous methods,

we believe that this method will widen the range of applicability of the microspectrographic procedures, in the first line on problems concerned with nucleic acid and protein metabolism. It is now technically possible to accumulate *absorption data representative not only for an entire inhomogeneous cell but also for a statistically significant numbers of cells within a relatively short time.*

REFERENCES

1. Caspersson, T., *Exp. Cell. Res.*, **1**, 595 (1950).
2. —— Cell growth and cell function. W. W. Norton, 1950, p. 38.